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GROWTH AND SURVIVAL OF *V. CHOLERÆ*, WITH SPECIAL REFERENCE TO GROWTH AND SURVIVAL IN WATER.

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Part I.

CONDITIONS AFFECTING MULTIPLICATION AND SURVIVAL OF *V. choleræ* IN ARTIFICIAL WATERS.

THE question of the relationship of water-supplies to the epidemiology of cholera has been the subject of consideration by most workers on this disease since it was shown in the Hamburg and London epidemics that contamination of the water-supply could be directly incriminated. The following is a brief résumé of recent work on this subject and other allied subjects :—

Panayotatau (1913) dealt with the question of the survival of cholera vibrios in the presence of other organisms. Schöbl (1914) gives figures for survival in tap water and sea water inoculated with rice-water stools. Panganiban and Schöbl (1918) pointed out the preservative action of salt on stools artificially inoculated with *V. choleræ*. Flu (1921) on the other hand was unable to demonstrate any preserving action of sea water on the cholera vibrios. Kabelik and Freudmann (1923) decided there was a relationship between the reaction of the medium and the optimum salt content for growth. They also found that salt facilitated the growth of *V. choleræ* as compared with *Bact. coli*, when mixtures were inoculated into weakly alkaline peptone water. Tohyama (1925) studied survival in salt solutions up to 30 per cent (saturated) NaCl. Yasuhara (1926) studied survival in

different natural waters. Khan (1930) reports amongst others eleven experiments on Ganges water heated at different temperatures. The inoculum was heavy and survival exceeded 5 days in two experiments only. d'Herelle, Malone and Lahiri (1930) summarize the literature and describe a few experiments on natural waters, mostly unheated in non-endemic areas. Their results do not support the idea of long survival in water. Tohyama and Yasukawa (1930) studied the survival of *V. metchnikovi* at different seasons of the year in sea water and give analyses of the water. Other Japanese workers, summarized by Tohyama and Yasukawa (*loc. cit.*), studied survival in sea water, while Yasukawa (1933) showed that the vibrio survived 22 days on the surface and 30 days at the bottom of an artificial sea-water tank, but that sterilization was effected by sunlight in two hours. The most favourable concentration of salt in sea water was 1·82 per cent. He also found that magnesium chloride had a more favourable influence on the viability of the cholera vibrio than any other salt.

In general a study of the above papers will show that, with the exception of Flu notably, most workers are agreed that the presence of salt in water facilitates the survival of the cholera vibrio. Very little attention has however been paid to the question of the multiplication of the vibrio in water which, from the point of view of the epidemiology of the disease, has possibly a greater importance.

An attempt has been made in the experiments to be described in this paper to establish the conditions under which *V. choleræ* may be expected to multiply and survive and thus to throw some light on factors that will have to be studied in the investigation of cholera infection of water in nature. It is fully realized that working with artificially inoculated solutions made up under laboratory conditions only very approximate reduplication of the conditions in nature can be obtained. It is, however, to be expected that, in the absence of factors which are found essential for or adjuvant to multiplication under laboratory conditions, increase in number of vibrios is unlikely in nature, where the conditions may be presumed to be ordinarily less favourable owing to the influence of sunlight, change of temperature and physical conditions, the presence of other organisms, etc.

The following factors were studied :—

Salt concentration.
Organic matter content.
Hydrogen-ion concentration.
Number of *V. choleræ* present in inoculum.
Action of other organisms commonly present in water and stools.

Salt concentration.

This was chiefly studied by adding to the medium employed an artificial concentrated sea-water solution made up to the following formula :—

		Parts.
NaCl	.. ..	27
MgCl$_2$-6H$_2$O	.. .. ..	3
KCl	.. ..	1
MgSO$_4$-7H$_2$O	.. .. ..	1·75
Aqua distillata	.. .. ..	100

This was added to the medium so that the final concentration of total salts varied between no salt and 3·0 per cent of the salt mixture, the small error due to the water of crystallization of the magnesium salts not being allowed for.

The percentage of chlorides in sea water is approximately 2·7.

Organic matter content.

A rough estimation of the organic matter present was obtained by determining the oxygen absorption from permanganate by Tidy's process. This method is of course ' strictly speaking, an index neither to the quantity nor to the quality of the organic matter ' (Thresh *et al.*, 1933). It is however similar in principle to that recommended by Asheshov *et al.* (1933) for the standardization of media. No difference was found between results obtained when various substances were used as bases for the media, which is in itself a justification for the applicability of this method to the problem in hand (Table IX). Peptone media were employed in most of the experiments as a convenient source of energy.

Hydrogen-ion concentration.

The pH was standardized for all ordinary experiments at between 7·6 and 8·2. There was no difference of growth characteristics between these limits ; in fact growth usually proceeded from an initial pH outside these limits till the medium became 7·8 to 8·0, when it remained stable. A special experiment is described to show the effect of reaction outside the normal limits.

Number of vibrios present in the inoculum.

The size of the inoculum was the subject of a great deal of preliminary experiment and an inoculum was finally adopted that was found to show differences to the best advantage. The effect of higher inocula was also examined in the study of survival.

Action of other organisms commonly present in stools and water.

The effect of concurrent presence of *Bact. coli*, *Bact. aerogenes*, *Proteus* and inagglutinable vibrios, *Streptococcus faecalis* and a gram + coccus isolated from water were also studied.

The technique employed was as follows :—

Peptone* solution or solutions of other organic bases were prepared in glass-distilled water in such concentration that the Tidy figure† as estimated by the method described by Thresh *et al.* (*loc. cit.*) was in the region of 0·3 parts per 100,000 of O_2 absorbed in 3 hours at 37°C. It was then easy, taking this as unit, to make

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* The peptone employed was Witte's peptone. On analysis the total inorganic substance after ashing in the muffle furnace was 2·2 per cent. This contained no chloride. The chloride present before ashing was 1·3 per cent estimated as NaCl by the method described by Husband and Godden (1927) for the estimation of chlorine in foodstuffs.

† All Tidy figures in this paper are the difference between readings at 3 minutes and 3 hours after incubation at 37°C.

solutions of higher and lower concentrations. To such solutions were then added the required amount of artificial sea-water solution. These were autoclaved in tubes and flasks or screw-capped bottles in different quantities according to the nature of the experiment. The reaction was adjusted to the region of 7·8 pH by the addition of small quantities of NaOH solution under aseptic conditions. It was then found that the pH did not materially change either from carbon-dioxide absorption or the growth of *V. choleræ*.

The inoculum consisted of a diluted overnight broth culture of *V. choleræ* of smooth Inaba type except in such cases as the amount of organic matter in the broth inoculum would have affected the total quantity of organic matter in the media, in which case agar washings in normal saline were substituted, controlled by bacterial counts, so that approximately the same number of viable organisms were introduced as would have been present in the broth inoculum. The standard inoculum chosen consisted of 2 c.c. of overnight broth culture of *V. choleræ* diluted to 1/10,000 by serial dilutions in normal saline per 100 c.c. of media. This was found to represent approximately 1,400 organisms per c.c. of media by viable count. Where agar washings were employed it was found that of a washing of the opacity of No. 3 Brown's tube, diluted 1/1,000, 0·15 c.c. per cent gave the same count.

The size of the inoculum was then tested by inoculating 0·2 c.c. of a series of decimal dilutions from the media under investigation into 5·5-c.c. papain-digest broth, prepared according to Asheshov's method (*loc. cit.*) with a pH of 7·6. The last tube in which growth occurred within 48 hours was noted, the culture plated out for colonial purity, and the colonies tested by slide agglutination against an 'O' serum diluted 1/100 (titre 1/2,500). With the standard inoculum growth occurred in most cases up to a dilution from the original inoculum of 1/100. A limit of error consisting of not more than 10 times larger and smaller, i.e., a one tube difference, has been allowed. A similar procedure was repeated at 24 and 48 hours and the limit of growth determined. Many similar experiments were carried out at 4 hours, 8 hours, and 3 days, but in most cases no additional information was obtained.

Multiplication was considered to have occurred when growth was recorded from 0·2 c.c. in at least 1/10,000 dilution.

Survival was tested by continuing incubation at 37°C. and examining daily from the undiluted culture by adding 0·2 c.c. to 5·5 c.c. papain broth up to 7 days, when the examination was then continued weekly, until no growth occurred at least 3 times in succession. It will thus be clear that survival as reported means survival in 0·2 c.c. of the media.

Effect of variation of salt concentration.

The results are given in Table I and summarized in Table II.

In the absence of salt multiplication did not occur in any peptone concentration and in no case did survival reach 24 hours. In 2 per cent sea salt multiplication was recorded except when peptone was under 1/500,000 dilution and in every experiment except one proceeded with survival extended to weeks or months under

the same conditions. In the intermediate salt concentrations no multiplication was recorded in 1/500,000 peptone below 1 per cent nor below 0·5 per cent sea salt in 1/50,000 (two experiments excepted), nor below 0·1 per cent in 1/5,000 (one experiment excepted). With 1/500 peptone multiplication took place in 0·075 per cent sea salt but not in 0·05 per cent. It will be noted that survival to a marked extent is correlated with initial multiplication.

The action of certain individual salts is given in Table VI. It is clear that any one of the salts tested except magnesium sulphate can promote multiplication, but none seems to do so in any specially low concentration. It was in an endeavour to elucidate the latter fact that the concentration of 0·1 per cent salt and peptone 1/5,000 was employed. The sea-water solution has somewhat higher capacity for promoting multiplication than the individual salts tested.

Many experiments were done with magnesium sulphate at many intermediate concentrations between 0·005 and 1 per cent but no evidence that this salt will promote multiplication or survival was obtained. In the higher concentration tested it is rapidly lethal to the vibrio.

Some interesting results of the effect shown by Na_2S in different concentrations are given in Table VII. Allowance has been made for the amount of sodium chloride formed when neutralizing the medium to a pH of 7·8. It will be observed that in 1/50,000 peptone the presence of 0·0003 per cent Na_2S in solution secured multiplication with 0·05 per cent of sea salt, while 0·00003 per cent was sufficient in 0·1 per cent sea salt. A reference to Table I will show that in only 1 out of 8 experiments did multiplication occur in the absence of Na_2S in these salt concentrations.

Effect of variation of organic matter content.

· It will be clear from what has been said on the effect of variation of salt content that variation in the organic matter content has little effect on the viable count, until the dilution exceeds 1/50,000 peptone. This is in accordance with Bail's hypothesis. The viable counts averaged growth in 0·2 c.c. in dilution of 10^{-7} to 10^{-9} in peptone solutions of 1/500 and upwards, 10^{-6} to 10^{-8} in 1/5,000, 10^{-5} and 10^{-6} in 50,000 and 1/500,000 in the presence of adequate salt content. The total count was of course vastly increased in the lower peptone dilutions. In dilutions of peptone higher than 1/500,000 no multiplication was obtained.

At a Tidy figure of 0·3 parts per 100,000 there was no difference in the growth or survival when soil extract, fæcal extract or carrot juice was employed as organic base (Table IX).

Effect of alteration of pH.

This was examined in 1/100 peptone with 1 per cent NaCl in stoppered bottles lined with paraffin wax. The range and results are indicated in Table X. It will be seen that full multiplication took place from 6·0 to just in excess of 9·4, though there was some reduction from 9·4 upwards. A pH of 9·2 may, therefore, be taken

as the limit for satisfactory multiplication, a figure which is supported by the results of experience in isolating the vibrio from natural sources. An experiment done in 1/50,000 peptone and 2 per cent sea salt is also appended. There is no essential difference from the results in 1/100 peptone, though the salt content being different the results are not strictly comparable.

Effect on survival of increasing the inoculum.

The results of experiments indicating this are given in Tables IV and V. The inoculum in all cases was equal to the maximum vital count obtainable in adequate salt concentration. Survival did not exceed 3 days in 1/5,000 peptone in the absence of salt. In 1/5,000,000 peptone it did not exceed 4 days in 0·01 per cent salt : in 0·02 per cent, however, it survived from 2 to 3 weeks and survival over 5 days was regular in 0·05 per cent even in the absence of organic matter (one experiment excepted in which the vibrio survived for five days).

Action of other organisms commonly present in stools and water.

Counts were carried out by the method of Anderson and Stuart (1935) except that standardized dropping pipettes were employed to drop the diluted emulsion uniformly over the plate before spreading by rotation.

Table XI gives the results in logs of counts per c.c. Absence of growth means absence from 0·2 c.c. on a countable plate. It will be seen that the agglutinable vibrio can survive in weak peptone and salt solutions even when in smaller inoculum, except in the presence of certain inagglutinable vibrios.

In several experiments it survived for two weeks or more in the presence of the latter vibrios. Where the organic pabulum is high, its survival period is sometimes shorter.

DISCUSSION.

In the absence of a definite quantity of salt and organic matter neither multiplication nor survival can take place. The quantity of organic matter required is low provided adequate salt is present. A peptone strength of 1/500,000 with a Tidy figure of 0·03 parts per 100,000 allows of multiplication in the presence of adequate salt, while a dilution ten times higher will not. The quantity of salt required is decreased with increasing strengths of organic matter, and in no case can multiplication take place in the absence of salt.

No one particular type of organic matter or salt with the possible exception of Na_2S was shown to have any action peculiar to itself. A number of salts have given similar results and qualitative differences in the organic matter seem immaterial. A mixture of salts similar to those in sea water has a greater facilitating action than any of the individual salts tried. Survival to a marked extent is coincident with initial multiplication. Provided the latter is adequate it may be expected that the organism will survive for many days. When the inoculum is largely increased a much lower concentration of salt is effective in producing survival.

The presence of other organisms in comparatively equal inocula with the exception of certain inagglutinable vibrios does not seem to prevent the multiplication and survival of *V. choleræ* up to 5 days or longer.

Part II.

APPLICATION OF ABOVE RESULTS TO NATURAL CONDITIONS.

As already pointed out, any deductions from the evidence presented must be subject to the very important consideration that no attention in the experimental work has been paid to physical factors such as sunlight, change of temperature, heavy monsoon rain, 'phage, etc., which are likely to have a profound effect.

Available figures of natural Indian waters showing oxygen absorption and salt content.

Clemesha *et al.* (1909) give analyses of waters in the Madras Presidency for the last quarter of the year 1908 and first quarter of 1909. The analyses show total solids estimations varying from 0·009 to 0·068 per cent, chloride estimations* 0·001 to 0·019, and Tidy figures of 0·004 to 0·320, there being little difference between the three classes of sources examined, rivers, surface waters, springs, and wells. Stewart and Boyd (1928) show figures varying from total solids 0·002 to 0·064 per cent, chlorides 0·001 to 0·010 per cent and Tidy 0·001 to 0·060, as ' illustrative examples ' of chemical analysis of Indian waters.

Were these figures typical of all Indian conditions the experimented evidence cited in this paper would not support the probability of vibrio multiplication or survival in water under most natural conditions. There is reason, however, to believe that the above figures are too low for many natural waters in Bengal. Two series of examinations of Bengal waters have been made by one of the authors, the first during a tour of Bengal in October 1937, when the monsoon effects were still present, when chloride estimations of different waters were made by the rough method indicated by Christophers, Sinton and Covell (1936). Chloride figures varying from 0·001 to 0·083 per cent were found in 12 samples in Burdwan district, which is on the border of Bengal, and removed from the deltaic area ; 0·001 per cent to 0·021 per cent in 13 samples from rural areas outside Calcutta and one figure of 0·173 per cent in a disused well, 0·002 per cent to 0·248 per cent in 28 samples in Jessore district, which is in the deltaic area, including one village, which showed in three separate tanks 0·107 per cent, 0·248 per cent, and 0·124 per cent ; and 0·002 per cent to 0·066 per cent in Khulna district, which is in the deltaic area adjacent to the sea. From these figures it may be concluded that, as other salts than chlorides will have been present in the water, that certain of the tanks contained inorganic matter at a figure suitable for vibrio multiplication. The figures for these two districts are given in Table XII.

Table. XIII shows the result of examination of tanks in the suburbs of Calcutta in April and May 1938, i.e., in the dry season of the year. In 32 tank samples the total solid figures were 0·1 per cent or over. The Hooghly river had

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* Calculated as sodium chloride from the Cl figures given.

the same figure and a back-water canal at one place was recorded as 1 per cent. The organic matter was in every case adequate for multiplication. From 4 samples the agglutinable vibrio was actually isolated.

Number.	Chlorides, per cent.	Total solids, per cent.	Tidy parts per 100,000.
24	0·046	0·10	0·96
43	0·041	0·1	1·52
52	0·026	0·08	0·55
53	0·014	0·11	0·74

N.B.—In no case, in spite of several attempts, could the isolation be repeated.

Multiplication and survival of agglutinable vibrio in autoclaved samples of natural Indian waters (Calcutta tanks).

In the case of samples shown in Table XIV 200 c.c. of each was autoclaved and inoculated with the standard inoculum of agglutinable vibrio, the pH being adjusted to 7·8 as the effect of autoclaving was to render these waters very alkaline. The pH of all the samples before autoclaving was suitable for vibrio multiplication. About six samples were examined in each month. The extent to which these samples were taken from the same tanks can be gathered from Table XV. Table XVI analyses the results of Table XIV as far as multiplication is concerned.

The total solids figure varied from 0·03 to 0·16 and the Tidy figure from 0·05 up to 3·1. It will be seen that the minimum figure coincident with multiplication differed at different seasons of the year. With a total solids figure of 0·06 and under no multiplication occurred in 13 specimens examined between June and October. Of 8 specimens examined in November and December 6 showed multiplication at this concentration, the two tanks which showed no multiplication being those that gave this result regularly throughout the year in spite of increase in the total solids figure through evaporation at a later period. (In one of these tanks multiplication was obtained with a total solids figure of 0·15 per cent upwards.) With a concentration of 0·07 one specimen each in May and August and five in December and January showed multiplication and one each in September and October no multiplication, one of the latter being a tank that regularly showed no multiplication; with a concentration of 0·08 and over of 27 specimens examined throughout the year 5 were negative, one in September, two in January, and one each in February and March, the four latter being from tanks that regularly failed to multiply. Survival over five days occurred in all except three specimens in September and October even from a total solids figure of 0·04 per cent and upwards. As might be expected owing to the incidence of the monsoon the average total solids figure tended to drop from June onwards till October, a rise being recorded from November onwards.

The Graph shows the mortality rate of the year in question as compared with the median mortality rate over 13 years.

GRAPH.

——————— Cholera deaths in Calcutta by 4-weekly periods, 1938–1939.

— — — — — Median cholera deaths in Calcutta over 13 years by 4-weekly periods.
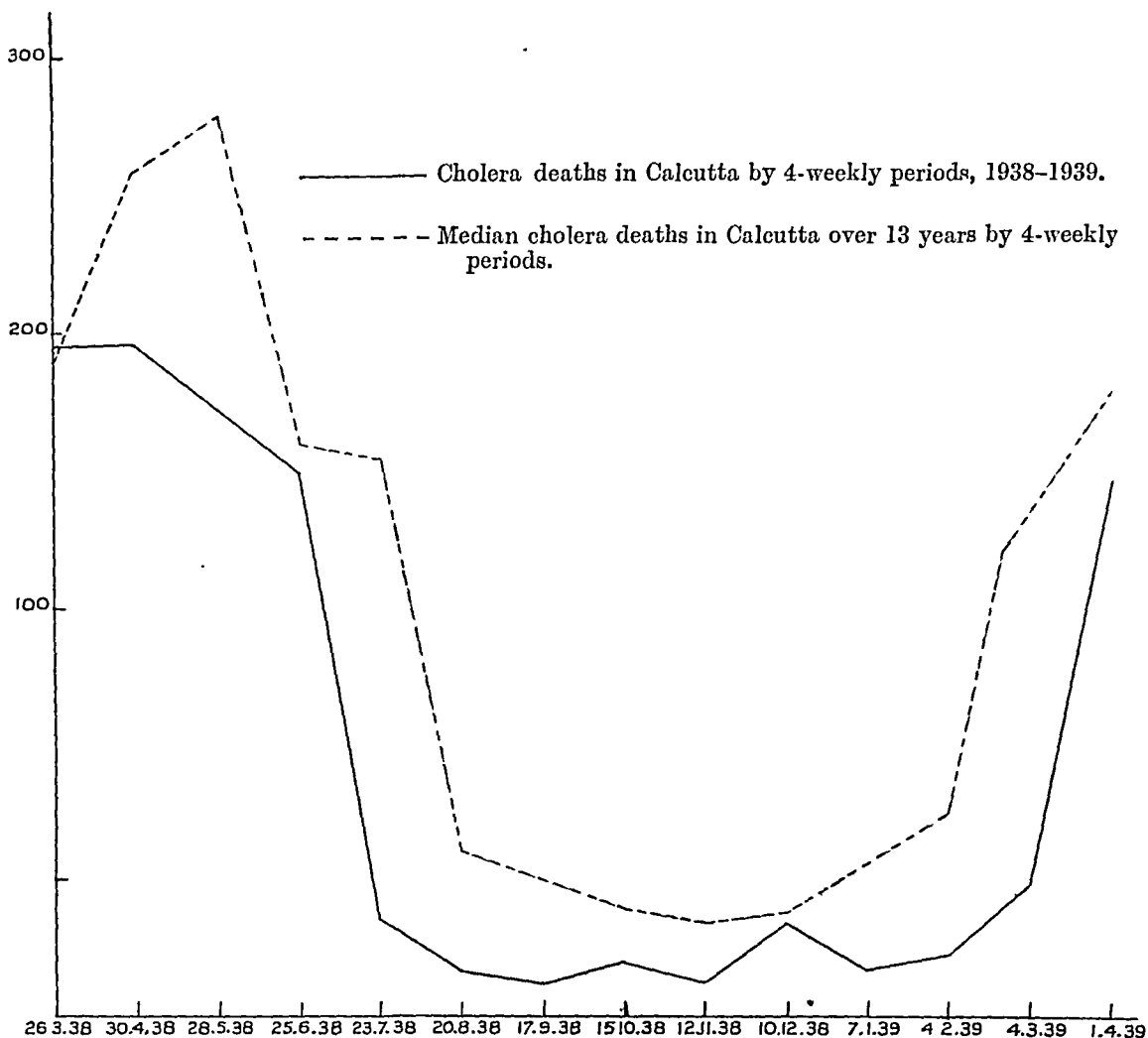
DISCUSSION.

The organic matter content as indicated by the Tidy figure would classify many of the Bengal waters as comparable with a peptone solution from 1/300,000 to 1/5,000, while the salt content as indicated by the total solids figure varied from an average of 0·05 to 0·1. Comparing the results in these natural waters with the experimental results in peptone solution it will be seen that in 1/50,000 peptone with 0·1 per cent sea water multiplication was obtained once only in five experiments with a survival period of over 7 days, though on one other occasion also survival up to 5 days was recorded. In 1/5,000 peptone with the same salt figure both

multiplication and survival were recorded in both of two experiments and in one of six experiments with 0·05 per cent sea-water solution. It therefore appears that slightly better conditions to promote multiplication may exist in the natural than in the artificial waters. On the other hand this may not always hold as one tank on two separate occasions failed to allow multiplication with a total solids figure of 0·1 per cent and Tidy figure of 0·17 and 0·52 respectively but this tank also was one in which multiplication was not obtained at any time throughout the year. Also as the results in Table VII show the experimental waters can under certain conditions equal the results in natural waters. None of the natural waters however gave a test positive for H_2S though the test employed would detect as little as 0·0001 per cent.

A further point emerges from a consideration of the details of the individual tanks examined. There is only a slight rise if any in the total solids figure from October to November and yet in five out of six cases examined in these two months multiplication failed to occur in October, while samples from the same tank allowed multiplication in the latter month. In the sixth in which multiplication did not occur no multiplication occurred at any period of the year, even when the total solids figure rose to 0·1 per cent. As Table XV shows previous to November multiplication was not obtained at a figure below 0·7 per cent but in November and December it was obtained on 6 occasions at 0·6 per cent or below. These facts require confirmation but they suggest that a qualitative change occurred in the samples examined. This change, if it does occur regularly, occurred at a period when the cholera death rate commences to increase.

While no deduction can justifiably be drawn at present from the above findings, especially in view of the fact that autoclaved samples were examined and the number examined was so inadequate, it appears that there is sufficient evidence to justify investigation on similar lines, where examination of natural sources in connection with cholera is being carried out.

SUMMARY.

1. Both salt and organic matter are required for the multiplication and survival of the cholera vibrio in artificially prepared water.

2. The amount of salt required decreases with an increase of strength of organic matter.

3. The conditions for multiplication and survival are very similar.

4. The presence of other organisms in comparatively equal inocula except the inagglutinable vibrio is not inimical to the multiplication of the agglutinable vibrio. In the presence of the inagglutinable vibrio the agglutinable vibrio can survive up to 3 weeks or longer.

5. There is a fairly close correspondence between the salt and organic matter required for multiplication in natural and in aitificial waters.

6. Available figures of analyses of natural waters in the Calcutta area suggest that the requisite conditions for multiplication and survival as far as salt content and organic matter are concerned are present in most open natural sources. There

is a general relationship between the average total solids figure of these waters and the prevalence of cholera.

The majority of the field work was carried out from the All-India Institute of Hygiene and Public Health, Calcutta, by the courtesy of Dr. R. B. Lal.

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TABLE I.

Growth and survival of V. cholerae in peptone and sea-salt solution.

Sea salt, per cent. → Peptone concentration ↓		Nil.	0·01	0·02	0·05	0·075	0·1
Nil.	Maximum growth	NG NG NG NG	‥	‥	‥	‥	‥
	Survival in days	0 0 0 0	‥	‥	‥	‥	‥
1/3,000,000	Maximum growth	NG	‥	‥	‥	‥	‥
	Survival in days	0	‥	‥	‥	‥	‥
1/500,000	Maximum growth	‥	‥	‥	‥	‥	NG
	Survival in days	‥	‥	‥	‥	‥	0

	1/50,000 { Maximum growth	NG	NG	NG NG	NG	..	NG NG	NG NG NG	NG	3	1	1	..	..	..	3	NG	NG	NG
	Survival in days	0	0	0	0	..	0	0	0	un	3	2	2	1	..	..	5	..	0	7–14
	1/5,000 { Maximum growth	NG	NG	NG NG NG	NG	..	NG	NG	un	1	5	NG	1	NG NG	3	2	3	6	6	..
	Survival in days	0	0	0	0	..	3	3	0	0	over 22	2	0	3	over 27	over 23	over 29	over 26	..
	1/500 { Maximum growth	NG	NG NG	NG	NG	..	NG	3	NG	NG	..	3	..	7	7	..	..	..	..
	Survival in days	0	0	0	..	..	0	over 26	0	0	0	over 26	over 19	..	..	..	..	..

NG = No growth in 0·2 c.c.
Maximum growth 2, 5, etc. = growth in 0·2 c.c. at a dilution of 1/10², 1/10⁵, etc.
'un' = growth in 0·2 c.c. in undiluted culture only.
Survival in days 'over 6' = experiment terminated after 6 days. Survival up to 6 days in 0·2 c.c.
    ,, ,, '6' = Survival to 6 days but not to 7.
    ,, ,, '6—13' = Survival to some undetermined period between 6 and 13 days.

TABLE I—concld.

Sea salt, per cent. / Peptone concentration.	Nil.		1/5,000,000		1/500,000	
	Maximum growth	Survival in days	Maximum growth	Survival in days	Maximum growth	Survival in days
0·2	..	..	..	..	..	..
	..	..	..	..	..	..
	..	..	..	..	..	..
	..	..	..	..	NG	0
	..	..	..	..	NG	0
0·5	..	..	..	..	..	..
	..	..	..	..	NG	0
	..	..	..	..	NG	0
0·75	..	..	..	..	..	..
1·0	..	..	..	..	NG	0
	..	..	..	..	6	over 27
	..	..	..	..	2	2
2·0	..	..	..	..	3	6
	..	..	1	1	6	over 28
	NG	0	NG	0	7	over 8
3·0	..	..	..	..	6	28—36
	..	..	..	..	5	over 28
	NG	0	..	..	un	2
	NG	0	NG	0	5	11

1/50,000	Maximum growth ..	un	3	3	NG	3	7	6	4	5	4	6	5	..	5	5	..	7	..
	Survival in days ..	1	over 28	over 26	0	1	over 26	28	7	over 26	over 29	over 27	over 29	..	over 39	26–33	..	over 64	..
1/5,000	Maximum growth ..	7	4	..	..	..	..	..	..	..	..	..	..	..	7	..	..	..	..
	Survival in days ..	over 64	over 28	..	..	..	..	..	..	..	..	..	..	..	over 47	..	..	..	..
1/500	Maximum growth ..	7	..	..	..	..	..	..	..	..	..	..	..	..	8	..	..	8	..
	Survival in days ..	over 144	..	..	..	..	..	..	..	..	..	..	..	..	over 47	..	..	over 57	..

NG = No growth in 0·2 c.c.
Maximum growth 2, 5, etc. = growth in 0·2 c.c. at a dilution of $1/10^2$, $1/10^5$, etc.
'un' = growth in 0·2 c.c. in undiluted culture only.
Survival in days 'over 6' = experiment terminated after 6 days. Survival up to 6 days in 0·2 c.c.
" " '6' = Survival to 6 days but not to 7.
" " '6—13' = Survival to some undetermined period between 6 and 13 days.

TABLE II.

Synopsis of Table I—Growth.

NUMBER OF EXPERIMENTS UPON WHICH RESULTS BASED.

Peptone concentration \ Sea salt, per cent.	Nil	0.01	0.02	0.05	0.075	0.1	0.2	0.5	0.75	1.0	2.0	3.0
Nil	4	..	..	..	..	..	..	..	..	..	1	2
1/5,000,000	1	..	..	..	..	..	..	..	..	..	2	1
1/500,000	..	..	..	..	..	1	2	2	..	2/1	2/1	3/1
1/50,000	5	1	2	3	..	4/1	5/1	3	1	2	2	1
1/5,000	6	..	2	5/1	2	2	2	..	..	..	1	..
1/500	4	..	..	3	2	..	1	..	..	..	1	1

MULTIPLICATION AS EVIDENCED BY TABLE I (FROM STANDARD INOCULUM).

Peptone concentration \ Sea salt, per cent.	Nil	0.01	0.02	0.05	0.075	0.1	0.2	0.5	0.75	1.0	2.0	3.0
Nil	—	—	—	—	—	—	—	—	—	—	—	—
1/5,000,000	—	—	—	—	—	—	—	—	—	—	—	—
1/500,000	—	—	—	—	—	—	—	—	—	⧺	⧺	⧺
1/50,000	—	—	—	—	—	⧺	⧺	+	+	+	+	+
1/5,000	—	—	—	⧺	—	+	+	+	+	+	+	+
1/500	—	—	—	—	+	+	+	+	+	+	+	+

Heavy type crosses refer to results obtained experimentally.

TABLE III.

Synopsis of Table I—Survival.

NUMBER OF EXPERIMENTS UPON WHICH RESULTS BASED.

Peptone concentration. \ Sea salt, per cent.	Nil	0.01	0.02	0.05	0.075	0.1	0.2	0.5	0.75	1.0	2.0	3.0
Nil ..	4	..	..	..	..	..	..	..	..	..	1	2
1/5,000,000	1	..	..	..	..	..	..	..	..	..	2	1
1/500,000	..	..	..	..	..	1	2	2	..	2/1	3	3/1
1/50,000	5	1	2	3	..	2/2	3/3	3	1	2	2	1
1/5,000	6	..	2	4/1	2	2	2	..	..	..	1	..
1/500	4	..	..	2/1	2	1	1	..	..	..	1	1

SURVIVAL OVER 5 DAYS AS EVIDENCED BY TABLE I (FROM STANDARD INOCULUM).

Peptone concentration. \ Sea salt, per cent.	Nil	0.01	0.02	0.05	0.075	0.1	0.2	0.5	0.75	1.0	2.0	3.0
Nil ..	−	−	−	−	−	−	−	−	−	−	−	−
1/5,000,000 ..	−	−	−	−	−	−	−	−	−	−	−	−
1/500,000 ..	−	−	−	−	−	−	−	−	−	++	+	++
1/50,000 ..	−	−	−	−	..	++	++	+	+	+	+	+
1/5,000 ..	−	−	−	++	+	+	+	+	+	+	+	+
1/500 ..	−	−	−	++	+	+	+	+	+	+	+	+

Heavy type crosses refer to results obtained experimentally.

TABLE IV.

Survival in peptone and sea salt from heavy inoculum.

Peptone concentration	Measurement	Nil.	0·01	0·02	0·05	0·075	0·1	0·2	0·5	2·0	3·0
Nil.	Minimum growth 48 hours.	1	··	NG; 5	6; 6	6; 5	5	··	6	5	6
Nil.	Survival in days ..	1	··	6; over 8	over 8; 5	over 27; over 8	over 27	··	over 21	over 28	over 28
1/5,000,000	Minimum growth 48 hours.	··	6; NG	7; NG; 5	5; 5	··	5	7	··	7	6
1/5,000,000	Survival in days ..	··	4; 0	14-21; 0; 7-15; over 6	over 7; over 14	··	over 27	over 7	··	over 28	over 28
1/500,000	Minimum growth 48 hours.	··	5	6; 5	5	··	··	8	··	··	··
1/500,000	Survival in days ..	··	4	over 29; 4	over 29	··	··	over 29	··	··	··

1/50,000 Minimum growth 46 hours.	..	..	..	5	un	5	5	2	..	..	..	..	..	6	..	7	..	..	..
Survival in days ..	..	..	..	over 21	2	6	over 21	2	..	..	..	..	..	over 28	..	over 31	..	..	..
1/5,000 Minimum growth 48 hours.	1	4	4	5	6	5	6	..	..	..	..	..	..	5	4	..	..	..	..
Survival in days ..	3	3	3	over 27	over 15	7–14	over 28	..	..	..	..	..	..	over 20	6	..	..	..	..
1/500 Minimum growth 48 hours.	7	7	..	..	..	..	..	..	..	..	5	..	..	..	..	..	..	..	..
Survival in days ..	over 28	over 29	..	..	..	..	..	..	..	..	over 25	..	..	..	..	..	..	..	..

Abbreviations as in Table I.

TABLE V.

Survival over 5 days as evidenced by Table IV and number of experiments in support.

Peptone concentration.	Sea salt, per cent.																			
	Nil.	0·01	0·02	0·05	0·075	0·1	0·2	0·5	2·0	3·0	Nil.	0·01	0·02	0·05	0·075	0·1	0·2	0·5	2·0	3·0
Nil	−	−	+	+	+	+	+	+	+	+	1	..	2	2	2	1	..	1	1	1
1/5,000,000	−	−	∓	+	+	+	+	+	+	+	..	2	$\frac{3}{1}$	2	..	1	1	..	1	1
1/500,000	−	−	∓	+	+	+	+	+	+	+	..	1	$\frac{1}{1}$	1	..	..	1	..	..	..
1/50,000	−	∓	∓	+	+	+	+	+	+	+	..	$\frac{2}{1}$	$\frac{1}{1}$	..	..	1	1	..	..	..
1/5,000	−	+	+	+	+	+	+	+	+	+	3	3	1	..	..	2	..	..	..	..
1/500	+	+	+	+	+	+	+	+	+	+	2	..	..	1	..	..	..	..	..	..

Heavy type crosses correspond to results obtained experimentally.
Light type ,, ,, ,, inferred.
Heavy type .. ,, ,,
Light type .. ,, ,,

TABLE VI.

Showing growth and survival in simple salts 0·1 per cent concentration and peptone 1/5,000 from standard inoculum of V. choleræ.

	MgCl$_2$	NaCl	KCl	CaCl$_2$	Na$_2$SO$_4$	NaNO$_3$	MgSO$_4$
Maximum growth.	NG, un, NG	NG, NG, 1	NG, NG, NG	NG, NG, NG	NG, un, NG	NG, un, NG	NG, un, NG
Survival in days.	0, 1, 0	0, 0, 1	0, 0, 0	0, 0, 0	0, 2, 0	0, 1, 0	0, 1, 0

Showing growth and survival in salts of 1 per cent concentration.

	MgCl$_2$	NaCl	KCl	CaCl$_2$	Na$_2$SO$_4$	NaNO$_3$	MgSO$_4$
Maximum growth.	6, 7, 4, 5	6, 7	6, 8, 6	4, 5, 4	4, 7, 6	6, 4, 5	NG, NG, NG
Survival in days.	over 8, over 13, over 8, 34	over 14, over 30	over 70, over 40, over 34	over 14, 18, over 28	over 70, over 24, over 66	over 27, over 28, over 28	0, 0, 0

Abbreviations as in Table I.

TABLE VII.

Showing growth and survival in sea salt, peptone, and sodium sulphide.

	Sodium sulphide, per cent.	0·00003				0·0003				0·003				
	Sea salt, per cent	0·05		0·1		0·02		0·05		0·02			0·05	
Peptone 1/50,000.	Maximum growth 48 hours.	NG	1	6	4	1	NG	6	5	NG	NG	NG	5	5
	Survival in days	0	1	over 28	10	1	0	over 28	6	0	0	0	over 7	10

Abbreviations as in Table I.

TABLE VIII.

Showing growth and survival over 5 days as evidenced by Table VII.

	Sodium sulphide, per cent.	0·00003	0·0003	0·003	NUMBER OF EXPERIMENTS.		
					0·00003	0·0003	0·003
Peptone 1/50,000.	Sea salt, per cent 0·02	..	—	—	..	2	3
	0·05	—	+	+	2	2	2
	0·10	+	..	..	2	..	..

.. = Experiment not done.

TABLE IX.

Growth on organic bases other than peptone.

Organic base.	Tidy parts per 100,000.		SEA SALT, PER CENT.				
			Nil.	0·2	0·5	1·0	2·0
Fæcal extract ..	0·369	Maximum growth	NG	16	..	..	7
		Survival in days	0	over 18	..	..	over 79
	0·387	Maximum growth	..	3	6	5	6
		Survival in days	..	2	4	over 19	over 19
	0·387	Maximum growth	..	3	6	6	6
		Survival in days	..	over 14	over 6	over 13	over 14
	0·427	Maximum growth	..	3	6	7	7
		Survival in days	..	15	15	over 21	over 21
Soil extract ..	0·306	Maximum growth	NG	4	..	..	4
		Survival in days	0	over 24	..	..	236
Carrot juice ..	0·354	Maximum growth	..	..	..	..	5
		Survival in days	..	..	..	..	over 5
	0·354	Maximum growth	..	..	..	..	6
		Survival in days	..	..	..	..	over 8
	0·354	Maximum growth	..	..	..	..	6
		Survival in days	..	..	..	..	64

Abbreviations as in Table I.

TABLE X.

Logs of counts at different pH.

Peptone, 1/100 NaCl, 1 per cent in 20 c.c. fluid in wax-lined stoppered bottles.

Initial pH	5·6	6·0	6·4	6·8	7·6	8·6	9·0	9·4	..	..	..	..
Drops dilute HCl added	55	46	38	30	0	..	..	..	..	..	..	..
Drops dilute NaCl added	..	..	..	..	..	10	40	60	80	100	120	140
Immediate count	2·70	2·88	3·15	3·16	3·13	3·10	3·00	3·29	3·31	3·32	3·28	3·34
Count at 48 hours	4·07	8·67	8·62	8·48	8·83	8·87	8·76	7·50	7·52	2·20	NG	NG
Count at 72 hours	3·23	..	..	..	..	..	..	..	..	..	..	..

Peptone, 1/50,000 Sea salt, 2 per cent

Initial pH	5·4	6·0	7·0	8·6	9·2	9·6
Immediate count	1·85	1·95	2·20	2·15	2·30	2·49
Count at 24 hours	NG	3·18	5·65	6·74	5·75	6·03
Count at 72 hours	2·64	6·38	6·30	6·19	6·94	6·28
Final pH	6·8	6·8	6·8	7·8	8·4	8·6

Abbreviations as in Table I.

TABLE XI-A.

Showing logs of counts[1] of mixtures of organisms grown in peptone 1/50,000, sea salt 2 per cent in 200 c.c. open flasks, pH 7·8.

Day	V. cholerae	Bact. aerogenes	V. cholerae	Bact. coli	V. cholerae	Bact. coli	V. cholerae	Bact. coli	V. cholerae	Streptococcus fæcalis	V. cholerae	Proteus	V. cholerae	Water coccus	V. cholerae	Water coccus	V. cholerae	W 9 E	V. cholerae	W. K. O.
Inoculum	2·81	3·88	3·54	4·02	3·51	Uncountable	3·15*	6·80	2·88	6·27	3·08	3·74	3·15	2·65	3·54	5·82	3·06	3·81	3·15*	7·35
1	6·96	5·18			3·78	0·07	7·24	7·78	7·14	Nil	6·99	2·48	7·33	Nil	6·71	6·60	5·65	6·91	Nil	7·32
2	7·10	5·40	6·29	6·96			7·66	7·29	6·94	Nil	6·94	0·78	7·54	Nil	6·67	3·39	5·90	6·94	Nil	7·49
3																			Nil	7·18
4																			Nil	7·30
5	6·79	5·48	6·00	6·85	4·48	5·81			5·82	Nil	6·72	Nil			6·13	Nil	5·48	6·59		
6	6·58	5·78	5·70	6·60	5·74	6·43					6·33	Nil					6·22	6·13	Nil	7·04
7	6·48	5·24	7·27	7·18	5·60	6·56									4·83	Nil	5·11	5·60		
14	6·23	5·13	5·97	4·78			6·30	7·10	4·05	Nil	5·52	Nil			5·35	Nil	Nil	4·85		
21	5·46	4·46							5·11						5·93	Nil	Nil	3·44		
28			5·32	5·11	4·30	2·70			3·29	Nil										
35	2·60	3·46	5·23	Nil	4·33	Nil			Nil	Nil					5·51	Nil	Nil	3·01		
42	2·13	2·35	4·42	Nil						Nil					5·41	Nil	Nil			
49	2·53	3·00			4·37	Nil			Nil	Nil							Nil	2·82		
56	3·06	2·48	4·45	Nil	3·74					Nil							Nil	2·32		
63			3·32	Nil	3·23	Nil												2·04		
70	3·54	2·00	3·23	Nil	2·86	Nil												2·05		
77	2·49	1·00	Nil	Nil		Nil														
84			Nil	Nil	2·60															
91			Nil	Nil		Nil														
98																				
105																				
115																				
170	1·70	Nil				Nil														

Uncountable = too small to be counted.
Nil = absent in 0·2 c.c. on a countable plate.
The majority of vibrio organisms are described in Taylor, Pandit and Read (1937).
The strain of *V. cholerae* was TMCH 1,800/1 isolated in Calcutta on 25th May, 1936.
V. cholerae Manzai was a very recently isolated agglutinable vibrio.
W 9 E = Water vibrio Heiberg II.
Other abbreviations as in Table I.
* Computed counts.

TABLE XI-A—concld.

Day	V. cholerae.	W. K. O.	V. cholerae.	PIS 984.	V. cholerae.	PIS 984.	V. cholerae.	L. I.	V. cholerae.	L. I.	V. cholerae.	Rangoon Rough.	V. cholerae.	Rangoon Rough.	V. cholerae Manzai.	L. I.	V. cholerae Manzai.	Rangoon Rough.	V. cholerae Manzai.	PIS 984.
Inoculum	2·40	3·18	3·15*	7·23	2·98	2·99	3·15*	7·30	2·85	2·70	3·15*	6·90	2·85	2·85	2·38	2·93	3·44	2·00	3·30	3·02
Day 1	*Nil*	6·06	4·60	7·49	5·00	6·41	5·30	7·29	6·66	5·78	*Nil*	7·32	6·00	5·95	7·22	7·15	7·36	5·30	7·48	6·11
„ 2	*Nil*	6·00	5·90	6·86	5·30	6·51	6·22	6·46	6·29	5·65	*Nil*	6·93	5·70	5·00	6·86	6·62	7·16	5·00		
„ 3	*Nil*	5·90		6·38	5·30	6·40		6·34	6·32	5·74			5·84	5·77						
„ 4	*Nil*	5·79	6·70		5·00	6·34	6·74		6·04	5·30	*Nil*	5·88	5·85	5·90						
„ 5	*Nil*	5·89							6·04	5·90			4·48	6·28						
„ 6	*Nil*	5·67	6·23	6·42	4·60	5·26	6·18	6·45	5·90	6·08			4·00	6·36	5·48	6·85	6·18	6·13	6·70	5·18
„ 7	*Nil*	4·85	6·67	5·00	4·78	5·60	*Nil*	5·37	5·86	5·23			4·48	5·08	5·00	6·36	5·53	5·44	6·24	5·90
„ 14								5·26									5·00	5·40	6·62	5·81
„ 21							*Nil*	5·02							*Nil*	6·31				
„ 28							*Nil*													
„ 35																				
„ 42																				
„ 49																				
„ 56																				
„ 63																				
„ 70																				
„ 77																				
„ 84																				
„ 91																				
„ 98																				
„ 105																				
„ 115																				
„ 170																				

Uncountable = too small to be counted.
Nil = absent in 0·2 c.c. on a countable plate.
The majority of vibrio organisms are described in Taylor, Pandit and Read (1937).
The strain of V. cholerae was TMCH 1,800/1 isolated in Calcutta on 25th May, 1936.
V. cholerae Manzai was a very recently isolated agglutinable vibrio.
W 9 E = Water vibrio Heiberg II.
Other abbreviations as in Table I.
* Computed counts.

TABLE XI-B.

Showing logs of count of mixtures of vibrios grown in peptone water 1/100, sea salt 0·5 per cent in 200 c.c. open flasks, pH 7·8.

	TMCH 353/3.	V. cholerae (TMCH) 1,800/1.	Rangoon Rough.	V. cholerae (TMCH) 1,800/1.	TMCH 1,805/1.	V. cholerae (TMCH) 1,800/1.	PIS 984.	V. cholerae (TMCH) 1,800/1.	I. 1.	V. cholerae TMCH 1,800/1.	W 9 E.	V. cholerae (TMCH) 1,800/1.	W 9 E.	V. cholerae (TMCH) 1,800/1.
Inoculum	2·40	2·36	1·70	2·60	2·83	2·88	2·13	2·59	1·40	2·54	3·92	8·56	2·48	2·40
Day 1	8·81	8·18	..	..	9·05	7·81	8·52	8·33	8·82	8·02	8·08	7·80	9·08	8·26
,, 2	8·40	7·70	8·00	8·30	8·02	7·90	8·56	7·93	8·76	7·48	8·27	7·84	9·18	8·04
,, 3	8·30	7·74	7·78	7·74	8·00	7·60	8·91	7·74	8·53	Nil	8·67	7·40	8·90	Nil
,, 4	8·76	Nil	6·60	7·38	7·29	6·40	8·09	7·02	8·31	Nil	8·31	Nil	8·98	Nil
,, 5	8·30	Nil	6·80	7·10	7·10	6·32	6·88	6·88	8·30	Nil	8·30	Nil	8·70	Nil
,, 6	8·40	Nil	..	..	..	..	6·00	6·79	..	..	8·24	Nil	..	..
,, 7	..	..	..	..	..	..	6·00	6·00	..	..	..	..	..	..
,, 14	..	..	..	..	..	..	Nil	7·53	..	..	..	..	..	..

For description of organisms *see* Taylor, Pandit and Read (1937).

TABLE XII.

Chloride percentage figures of natural waters (October 1937),
post-monsoon period.

Sources.	Burdwan district.	Calcutta area (rural area outside Calcutta).	Jessore district.	Khulna district.
Rivers	0·002	0·002	0·010	0·002
	0·002	0·001	0·006	0·322*
	0·002	0·017	0·002	..
	0·001	0·021	0·002	..
Tanks and surface waters	0·007	0·009	0·002	0·008
	0·011	0·021	0·008	0·010
	0·005	0·012	0·025	0·008
	0·017	0·007	0·010	0·066
	0·016	0·008	0·012	0·033
	0·004	0·010	0·012	0·058
	..	0·008	0·007	0·033
	..	0·008	0·002	0·058
	..	0·008	0·007	0·033
	..	..	0·107	0·025
	..	..	0·248	0·066
	..	..	0·124	0·033
	..	..	0·014	..
	..	..	0·050	..
	..	..	0·013	..
	..	..	0·017	..
	..	..	0·025	..
	..	..	0·008	..
	..	..	0·008	..
	..	..	0·008	..

* Tidal.

TABLE XII—*concld.*

Sources.			Burdwan district.	Calcutta area (rural area outside Calcutta).	Jessore district.	Khulna district.
			0·083*	0·173*	0·003	..
			0·005	..	0·002	..
Wells	..	..	..	..	0·013	..
			..	..	0·020	..
			..	..	0·005	..

* Disused.

TABLE XIII.

*Chemical examination of open water sources in suburbs of Calcutta.
May-June* 1938.

	pH.	Chloride, per cent.	Total solids, per cent.	Tidy per 100,000 (difference of 3 minutes and 3 hours figures at 37°C.).
TANK NUMBER.				
9	8·0	0·008	0·03	0·94
10	7·2	0·010	0·04	0·97
11	7·1	0·003	0·05	0·88
12	8·2	0·016	0·05	1·26
13	8·0	0·016	0·06	0·57
14	8·2	0·061	0·09	0·24
15	8·4	0·018	0·06	1·51
23	8·2	0·066	0·13	2·27
24	8·2	0·046	0·10	0·96
28	8·2	0·047	0·09	0·71
39	8·1	0·043	0·09	0·52
48	8·0	0·033	0·09	0·59
60	8·0	0·031	0·08	0·67

TABLE XIII—*contd.*

TANK NUMBER.	pH.	Chloride, per cent.	Total solids, per cent.	Tidy per 100,000 (difference of 3 minutes and 3 hours figures at 37°C.).
25	8·4	0·036	0·10	1·00
26	8·5	0·038	0·10	2·40
27	8·0	0·052	0·11	2·79
30	8·6	0·048	0·10	1·00
31	8·0	0·048	0·10	2·13
32	8·8	0·035	0·09	0·92
33	8·0	0·082	0·16	1·89
34	8·2	0·064	0·13	1·48
35	8·8	0·013	0·05	0·95
36	8·2	0·044	0·09	0·22
37	8·0	0·020	0·08	0·96
38	7·5	0·013	0·05	0·67
40	8·4	0·042	0·09	0·38
41	8·8	0·034	0·09	1·24
42	8·0	0·033	0·08	1·20
43	8·0	0·041	0·10	1·52
49	8·2	0·033	0·09	1·11
59	8·0	0·029	0·08	1·82
75	8·2	0·033	0·09	1·09
44	7·8	0·014	0·05	0·32
45	8·3	0·034	0·10	0·94
46	8·4	0·024	0·08	0·43
47	8·2	0·016	0·06	0·41
50	8·8	0·023	0·08	0·61
51	9·4	0·016	0·05	0·38

TABLE XIII—*contd.*

TANK NUMBER.	pH.	Chloride, per cent.	Total solids, per cent.	Tidy per 100,000 (difference of 3 minutes and 3 hours figures at 37°C.).
52	8·2	0·026	0·08	0·55
65	8·2	0·026	0·07	0·60
53	8·2	0·044	0·11	0·74
66	8·2	0·033	0·10	0·77
54	8·4	0·020	0·07	1·64
55	8·2	0·048	0·12	0·82
56	9·0	0·023	0·08	0·94
57	8·6	0·051	0·13	1·50
58	8·6	0·072	0·17	1·41
51	..	0·043	0·11	0·75
62	..	0·018	0·06	0·47
63	..	0·016	0·06	0·70
64	..	0·031	0·08	0·46
67	..	0·016	0·05	0·31
68	..	0·087	0·17	0·80
69	8·6	0·033	0·09	0·86
70	8·0	0·013	0·05	0·44
71	8·4	0·026	0·08	0·56
72	8·6	0·056	0·13	0·97
73	8·1	0·013	0·02	0·67
74	8·6	0·011	0·04	0·59
76	..	0·026	0·09	0·54
77	8·0	0·048	0·11	0·90
78	8·2	0·026	0·08	0·07
79	8·1	0·018	0·06	0·39

TABLE XIII—*contd.*

TANK NUMBER.	pH.	Chloride, per cent.	Total solids, per cent.	Tidy per 100,000 (difference of 3 minutes and 3 hours figures at 37°C.).
80	8·0	0·053	0·10	0·85
81	8·4	0·015	0·06	0·21
82	8·0	0·024	0·07	0·15
83	7·9	0·029	0·08	0·03
84	8·0	0·041	0·09	0·09
85	9·2	0·046	0·10	1·24
86	8·2	0·072	0·13	1·02
87	8·0	0·066	0·13	0·81
88	9·0	0·031	0·08	2·22
89	8·2	0·050	0·12	0·93
90	8·8	0·038	0·10	0·99
91	8·4	0·044	0·10	1·02
92	8·6	0·033	0·08	·1·04
93	8·0	0·023	0·07	0·55
94	8·0	0·073	0·17	1·43
95	9·0	0·033	0·09	1·55
96	9·4	0·056	0·12	1·48

TABLE XIII—*concld.*

	pH.	Chloride, per cent.	Total solids, per cent.	Tidy per 100,000 (difference of 3 minutes and 3 hours figures at 37°C.).
TANK NUMBER.				
97	8·4	0·045	0·11	0·66
98	7·8	0·038	0·10	0·96
99	8·6	0·035	0·10	0·58
100	8·0	0·031	0·09	0·88
RIVER NUMBER.				
16	8·2	0·064	0·11	0·95
17	8·2	0·073	0·12	0·71
18	8·2	0·060	0·10	0·76
19	8·2	0·061	0·10	0·94
CANAL NUMBER.				
20	8·2	0·683	0·87	0·89
21	8·0	0·574	0·74	0·81
22	8·2	0·858	1·09	0·82
DRAIN NUMBER.				
29	7·6	0·066	0·14	2·18

TABLE XIV.

Showing multiplication and survival of V. choleræ in autoclaved samples of Calcutta tank water in different months of the year.

	MAY.			JUNE.									AUGUST.				
Number of sample.	54A	56	57	40	38	36	41	35	42	54	40	53	48	49	51	52	50
Total solids, per cent	0·07	0·08	0·13	0·05	0·06	0·08	0·09	0·10	0·12	0·05	0·05	0·06	0·06	0·07	0·08	0·08	0·10
Monthly average of total solids.		0·09						0·08							0·07		
Chloride, per cent	0·020	0·023	0·051	0·018	0·021	0·034	0·036	0·041	0·063	0·025	0·018	0·028	0·025	0·030	0·036	0·030	0·041
Average chloride, per cent.		0·031						0·036							0·029		
Tidy parts per 100,000.	1·64	0·94	1·50	0·59	0·42	0·43	0·27	0·35	0·48	0·27	0·59	0·07	0·31	0·24	0·05	0·15	0·36
Average Tidy		1·03						0·42							0·26		
Maximum multiplication.	4	4	5	1	2	5	6	5	4	3	1	2	1	5	6	5	5
Survival in days ..	over 12	over 12	over 12	over 28	over 14	over 21	over 14	over 7	over 7	over 28	over 28	7	over 7	over 17	over 17	over 17	over 30

Abbreviations as in Table I.

TABLE XIV—contd.

	SEPTEMBER.					OCTOBER.						NOVEMBER.					
Number of sample.	7	1	5	4	3	4/10	3/10	1/10	6/10	2/10	7/10	4/11	3/11	2/11	1/11	5/11	6/11
Total solids, per cent.	0·05	0·06	0·07	0·08	0·09	0·03	0·04	0·05	0·05	0·05	0·07	0·05	0·05	0·05	0·06	0·06	0·06
Monthly average of total solids.			0·07					0·05						0·06			
Chloride, per cent	0·033	0·031	0·030	0·035	0·043	0·011	0·021	0·025	0·028	0·024	0·028	0·021	0·021	0·023	0·028	0·033	0·031
Average chloride, per cent.			0·034					0·023						0·026			
Tidy parts per 100,000.	0·35	0·33	0·44	0·40	0·52	0·49	0·39	0·11	0·53	0·58	1·06	0·84	0·86	1·0	0·48	0·51	0·58
Average Tidy ..			0·41					0·53						0·71			
Maximum multiplication.	NG	NG	un	2	5	3	2	1	3	2	3	6	6	5	3	4	6
Survival in days ..	0	0	over 14	over 16	over 14	5	over 28	over 28	over 6	over 7	over 28	over 5	over 8	over 5	over 5	over 5	over 8

Abbreviations as in Table I.

TABLE XIV—contd.

	December					January					
Number of sample.	2/12	4/12	1/12	5/12	6/12	1/1	2/1	4/1	6/1	3/1	5/1
Total solids, per cent.	0·06	0·06	0·07	0·07	0·07	0·07	0·07	0·08	0·08	0·10	0·10
Monthly average of total solids.			0·07					0·08			
Chloride, per cent	0·033	0·028	0·029	0·039	0·031	0·022	0·022	0·032	0·041	0·051	0·042
Average chloride, per cent.			0·032					0·035			
Tidy parts per 100,000.	0·30	0·65	0·32	0·67	0·85	0·21	0·54	0·43	0·48	0·17	0·43
Average Tidy ..			0·56					0·38			
Maximum multiplication.	1	6	4	4	4	5	5	3	5	un	6
Survival in days ..	7–14	over 7	over 6	over 7	over 7	over 7	over 5	over 7	over 7	over 7	over 7

Abbreviations as in Table I.

TABLE XIV—*concld.*

	FEBRUARY.						MARCH.					
Number of sample.	6/2	2/2	1/2	4/2	3/2	5/2	1/3	3/3	2/3	6/3	4/3	5/3
Total solids, per cent.	0·08	0·08	0·10	0·10	0·11	0·16	0·10	0·10	0·11	0·12	0·12	0·15
Monthly average of total solids.	0·11						0·12					
Chloride, per cent	0·051	0·043	0·076	0·049	0·053	0·076	0·049	0·049	0·062	0·080	0·057	0·084
Average chloride, per cent.	0·058						0·064					
Tidy parts per 100,000.	0·6	1·3	0·52	1·07	1·6	3·1	1·4	1·4	1·1	0·9	2·2	1·3
Average Tidy ..	1·37						1·4					
Maximum multipli-cation.	4	6	2	5	7	5	5	7	6	1	7	7
Survival in days ..	over 5	over 5	over 5	over 5	over 5	over 5	over 7	over 21	over 14	7–14	7–14	7–14

Abbreviations as in Table I.

TABLE XV.

Showing multiplication and survival in autoclaved samples taken from individual Calcutta tanks month by month.

Month.	KALU BABU'S TANK.					GOLEPUKUR TANK.					MIAH'S TANK.					BYSAK BABU'S TANK.				
	T. S.	Chlor.	Tidy.	M.	S.	T. S.	Chlor.	Tidy.	M.	S.	T. S.	Chlor.	Tidy.	M.	S.	T. S.	Chlor.	Tidy.	M.	S.
1938.																				
June ..	0·10	0·041	0·35	+	+	..	..	..	..	..	0·08	0·034	0·43	+	+	0·06	0·021	0·42	−	+
July ..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
August ..	0·08	0·036	0·05	+	+	0·08	0·030	0·15	+	+	..	..	..	..	..	0·06	0·025	0·31	−	+
September ..	0·09	0·043	0·52	+	+	0·05	0·033	0·35	−	−	0·08	0·035	0·40	−	+	0·06	0·031	0·33	−	+
October ..	0·04	0·021	0·39	−	+	0·05	0·028	0·53	−	+	0·03	0·011	0·49	−	+	0·05	0·025	0·11	−	+
November ..	0·05	0·021	0·86	+	+	0·06	0·033	0·51	+	+	0·05	0·021	0·84	+	+	0·06	0·028	0·48	−	+
December ..	..	..	..	..	..	0·06	0·028	0·65	+	+	..	..	..	..	..	..	..	..	..	..
1939.																				
January ..	0·07	0·022	0·54	+	+	0·08	0·041	0·48	+	+	0·07	0·022	0·54	+	+	0·10	0·05	0·17	−	+
February ..	0·10	0·049	1·07	+	+	0·11	0·053	1·6	+	+	0·08	0·051	0·60	+	+	0·10	0·076	0·52	−	+
March ..	0·10	0·049	1·4	+	+	0·10	0·049	1·4	+	+	0·11	0·06	1·1	+	+	0·12	0·080	0·90	−	+

Note.—T. S. = Total solids, per cent.
      Chlor. = Chlorides, per cent.
      Tidy = Tidy parts per 100,000.
      M. = Multiplication.
      S. = Survival.

TABLE XV—concld.

Month.	Kamal Babu's Tank.					Mukundar Sikdar's Tank.					Prantosh Ganguli's Tank.					Satish Babu's Tank.				
	T.S.	Chlor.	Tidy.	M.	S.	T.S.	Chlor.	Tidy.	M.	S.	T.S.	Chlor.	Tidy.	M.	S.	T.S.	Chlor.	Tidy.	M.	S.
1938.																				
June	0·05	0·018	0·50	—	+	0·09	0·036	0·27	+	+	0·12	0·063	0·48	+	+	..	..	..	..	..
July	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
August ..	0·05	0·025	0·27	—	+	..	..	..	..	..	0·06	0·028	0·07	—	+	0·07	0·03	0·24	+	+
September ..	0·07	0·030	0·44	—	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
October ..	..	..	..	..	..	0·07	0·028	1·06	—	+	..	..	..	..	..	0·05	0·024	0·58	—	+
November ..	..	..	..	..	..	0·06	0·031	0·58	+	+	..	..	..	..	..	0·05	0·023	1·4	+	+
December ..	0·06	0·033	0·30	—	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
1939.																				
January ..	0·08	0·032	0·43	—	+	0·10	0·042	0·43	+	+	..	..	..	..	..	..	..	..	..	..
February ..	0·16	0·076	3·1	+	+	0·08	0·043	1·3	+	+	..	..	..	..	..	..	..	..	..	..
March ..	0·15	0·084	1·3	+	+	0·12	0·057	2·2	+	+	..	..	..	..	..	..	..	..	..	..

Note.—T. S. = Total solids, per cent.
Chlor. = Chlorides, per cent.
Tidy = Tidy parts per 100,000.
M. = Multiplication.
S. = Survival.

TABLE XVI.

Analysis of Tables XIII and XIV.

Month.	TOTAL SOLIDS, PER CENT.					TOTAL SOLIDS, PER CENT.				
	0·05 and under.	0·06	0·07	0·08	0·09 and over.	0·05 and under.	0·06	0·07	0·08	0·09 and over.
1938.										
May	..	..	+	+	+	..	..	1	1	1
June	—	—	..	+	+	1	1	..	1	3
July	..	..	..	..	..	..	..	..	..	..
August	—	—	+	+	+	2	2	1	2	1
September	—	—	—	—	+	1	1	1	1	1
October	—	..	—	..	..	5	..	1	..	..
November	+	±	..	..	..	3	$\frac{2}{1}$	..	..	..
December	..	±	+	..	..	..	$\frac{1}{1}$	3	..	..
1939.										
January	..	..	+	±	±	..	..	2	$\frac{1}{1}$	$\frac{1}{1}$
February	..	..	..	+	±	..	..	..	2	$\frac{3}{1}$
March	..	..	..	..	±	..	..	..	..	$\frac{5}{1}$

+ multiplication occurred.
— no multiplication occurred.
The right half of the table indicates the number of samples examined.

ON INHIBITION OF INDIVIDUAL TYPES OF CHOLERA-BACTERIOPHAGE BY VIBRIO EXTRACTS.

BY

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(*An Inquiry under the Indian Research Fund Association, Pasteur Institute and Medical Research Institute, Shillong.*)

In a previous communication by Pandit, Maitra and Datta Roy (1936) it was shown that extracts prepared according to the method of Burnet (1934) of typical cholera and allied vibrios, inhibited the lytic action of certain of the ten types, e.g., A, B, C, D, E, F, G, H, J, and K, of choleraphages used in that investigation, and that the vibrio strains whose extracts were investigated could be classified into three groups according to their 'phage-type inhibitions. It was also tentatively concluded that these groups corresponded in general to the grouping obtained by Linton and Mitra (1934-1935) according to the respective polysaccharides of those strains as determined by chemical analysis. The present communication is an account of the work which was followed in continuation of the previous studies with a few more biological data regarding inhibition.

Burnet (*loc. cit.*) observed that the 'phage inactivating power is destroyed by the addition of homologous anti-serum concurrently with the appearance of specific precipitin reaction. Precipitin sera were prepared from typical strains of inhibition groups I, II, and III vibrios, using boiled suspensions of the organisms. The strains selected were :—

1617—group I by inhibition test. Linton's carbohydrate I.

603—group II by inhibition test. Linton's carbohydrate III.

W 832—group III by inhibition test. A water vibrio, polysaccharide not known.

W 880—group III by inhibition test. Linton's carbohydrate II.

Rabbits were immunized against these strains by the intensive method described by Julianelle and Wieghard (1935). These sera were diluted 1 in 30, 1 in 25, 1 in 20,

and 1 in 20, respectively. Equal quantities of serum and extracts were allowed to remain in contact at 37°C. for two hours and the readings were then taken immediately. The formation of a ring at the surface of contact was taken as a positive reaction. The results are given in Table I :—

TABLE I.

Groups by inhibition.	Extracts.	PRECIPITIN REACTION AT 37°C. FOR TWO HOURS.			
		Serum 1617 1 in 30, group I.	Serum 603 1 in 25, group II.	Serum W 832 1 in 20, group III.	Serum W 880 1 in 20, group III.
GROUP I ..	653	+	+	—	—
	Inaba ..	+	+	—	—
	Ogawa ..	+	+	—	—
	1617 ..	+	+	—	—
	W 880 (S.T.M.P.)	+	+	—	—
	M 45 ..	+	+	—	—
	M 36 ..	+	+	—	—
	M 12 ..	+	+	—	—
GROUP II ..	R Recovered ..	+	+	—	—
	505	+	+	—	—
	603	+	+	—	—
GROUP III ..	1612 ..	—	—	—	—
	El Tor I ..	—	—	—	—
	W 880 ..	—	—	—	+
	W 832 ..	—	—	+	—
	1295 ..	—	—	—	—
	916 (H. B.) ..	—	—	—	—
	12 (H. B.) ..	—	—	—	—

+ = Positive.   — = Negative.

It will be seen from Table I, that extracts of vibrios of inhibition groups I and II all gave a positive precipitin reaction with both group I and group II sera and invariably a negative result with group III serum. On the other hand the group III sera gave a positive result with their homologous extract only and were negative with the extracts of all the other group III organisms, which were also not precipitated by groups I and II sera. An attempt was then made to separate by means of the precipitin test the vibrios of groups I and II, which appeared to be differentiated, though not very definitely, by the inhibition test. This, however, was not successful. Moreover, an 'O' serum, Inaba or Ogawa of inhibition group I, agglutinates the vibrios Inaba or Ogawa of inhibition group II and vice versa. This preliminary work suggested that vibrios of inhibition group III possess a marked antigenic individuality and that vibrios of inhibition groups I and II possess a common antigenic factor and that following Burnet this antigenic factor is a poly-saccharide present in the 'O' antigen of the vibrio. The inhibition groups I and II vibrios have the same 'A' 'phage type inhibitory substance present in their extracts, while the inhibition group III vibrios fail to inhibit type A. The specific substance responsible for the inhibition of type A by the extracts of groups I and II vibrios appears to be the common factor for the precipitation with both group I and group II sera. In the light of this finding our previous grouping of vibrios into three groups appears unnecessary. Inhibition groups I and II of our previous work are, therefore, for the present combined into one single group I, and the original group III now becomes group II. With this revised grouping the vibrios fell into two main groups, the typical cholera vibrios into group I and the atypical, inagglutinable strains and water vibrios into group II.

Continuing this work, inhibition tests were done with extracts of vibrios received from diverse sources. The extracts were prepared in the same way as reported in our previous paper and inhibition and precipitin reactions were performed to determine if any correspondence exists between agglutination, inhibition, and precipitin reactions. The results are shown in Table II.

From Table II it appears that a general correlation exists between 'O' agglutination, precipitation, and inhibition by extracts of group I. One exception will be noticed, i.e., strain M 47. This is not a typical agglutinable vibrio and yet by these tests it falls into inhibition group I. Strain M 47 was isolated among numerous other agglutinable vibrios in an epidemic of cholera in Manipur State (Assam) in 1935*.

Two of the El Tor vibrios belonging to Gardner's 'O' sub-group I were indistinguishable from the typical cholera vibrios by these two tests. Inaba 'rough' obtained by Bruce-White by specific bactericidal method also fell into inhibition group II by these tests.

Inhibition with extracts of cultures rendered resistant to 'phage types in the laboratory.—In working with cholera-bacteriophage, it was found (Morison, 1932) that, given n types of 'phage, a susceptible vibrio strain could be rendered resistant to these n types in combinations of n-1 at a time and also to all the types taken together

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* The strain M 47 was subsequently found to be a mixed culture from which the typical agglutinable vibrio was isolated. It appears that the inhibition and precipitin tests are even more sensitive than the agglutination test.

TABLE II.

Strains.	Action of bacteriophage types.		Agglutination by 'O' serum, per cent of titre.	Types of 'phage inhibited.	Inhibition group.	Precipitin reaction with an 'O' serum, group I.
	Lysed by	Resistant to				
F 147 ..	C, J	A, B, D, E, F, G, H, K, L, M.	100	A	I	+
F 148 ..	C, J	A, B, D, E, F, G, H, K, L, M.	100	A	I	+
F 85 (O. B.) ..	C, F, J, M	A, B, D, E, G, H, K, L.	100	A, B, F, H	I	+
M 47 ..	Nil	Nil	Negative	A, B	I	+
2027 ..	B, C, D, E, F, H, J	A, K, L	100	A, B, D. E, F, H, J	I	+
2029 ..	B, C, D, E, F, G, H, J, K, L.	A	100	A, B	I	+
El Tor 34/11 Gardner's group I.	A, D, E, H, J	B, C, F, K, L	100	A, B, D, F, H	I	+
El Tor 34/13 Gardner's group I.	Nil	All types	100	A, B, D	I	+
F 5 (7) ..	J	A, B, C, D, E, F, G, H, K, L.	100	A, B	I	+
F 5 (12) ..	C, E, F, H, J	A, B, D, G, K, L	100	A, H	I	+
F 15 (1) ..	C, J	A, B, D, E, F, G, H, K, L.	50	A	I	Not done.
F 70 (A) ..	B, C, D, E, F, G, H, J	A, K, L	50	A, B, F, H, J	I	,,
F 85 (A) ..	C, F, J	A, B, D, E, G, H, K, L.	100	A, F	I	,,
Etawah ..	Nil	All types	100	A	I	+

W 880 (S. T. M. P.) ..	A, B, C, D, E, F, G, H, J.	K, L	50	A, B, D, F, H, J	I	+
Inaba ..	A, B, D, E, F, G, H, J, K, L.	*Nil*	100	A, B, D, F, H, J, K	I	+
Inaba R ..	B, C, D, E, F, G, H, J, K, L.	A	Negative	*Nil*	II	−
857 (H. B.)	*Nil*	All types	,,	*Nil*	II	−
984 (H. B.)	*Nil*	,,	,,	*Nil*	II	−
12 (H. B.)	*Nil*	,,	,,	*Nil*	II	−
3065 (H. B.)	*Nil*	,,	,,	*Nil*	II	−
W 880 ..	*Nil*	,,	,,	*Nil*	II	Not done.
W 236 ..	*Nil*	,,	5	*Nil*	II	,,
W 239 ..	*Nil*	,,	12·5	*Nil*	II	,,
W 161 (2) ..	E, F, M	A, B, C, D, G, H, J, K, L.	Negative	*Nil*	II	,,
I. M. H. (Lucknow) ..	B, J	A, C, D, E, F, G, H, K, L.	,,	F, H	II	,,
1643 (Bangalore) ..	G	A, B, C, D, E, F, H, J, K, L.	,,	B, E	II	,,
1640 (Bangalore) ..	*Nil*	All types	5	*Nil*	II	,,
W 345 (1) ..	M, H	A, B, C, D, E, F, G, J, K, L.	Negative	*Nil*	II	,,
F 35 (1) ..	*Nil*	All types	,,	*Nil*	II	,,
W 27 ..	C, F	A, B, D, E, H, J, K, L.	,,	*Nil*	II	−
W 127 ..	F	A, B, C, D, E, G, H, J, K, L.	5	B, E, F	II	Not done.
U. P. V. ..	*Nil*	All types	Negative	B, D, H, J	II	,,

so that a series of multi-type resistant cultures could be obtained each of which was lysed by one only of the n types, whilst the n-resistant culture was not susceptible to the action of any type of bacteriophage then known to exist. Extracts were prepared with such multi-type resistant cultures according to Burnet's technique and inhibition tests were performed. The results are given in Table III :—

TABLE III.

Extracts.	Strains from which extracts prepared.	ACTION OF THE TYPES OF 'PHAGE.		Types of 'phage inhibited.	Inhibition group.	Precipitin reaction with an 'O' serum, group I.
		Lysed by	Resistant to			
$(11\text{-}A)^r$ ..	$(11\text{-}A)^r$	A	B, C, D, E, F, G, H, J, K, L.	A	I	+
$(11\text{-}B)^r$ ..	$(11\text{-}B)^r$	B	A, C, D, E, F, G, H, J, K, L.	Nil	II	—
$(11\text{-}C)^r$ ..	$(11\text{-}C)^r$	C	A, B, D, E, F, G, H, J, K, L.	Nil	II	—
$(11\text{-}D)^r$ ..	$(11\text{-}D)^r$	D	A, B, C, E, F, G, H, J, K, L.	D	II	—
$(11\text{-}F)^r$ ..	$(11\text{-}F)^r$	F	A, B, C, D, E, G, H, J, K, L.	Nil	II	—
$(11\text{-}G)^r$ ..	$(11\text{-}G)^r$	G	A, B, C, D, E, F, H, J, K, L.	Nil	II	—
$(11\text{-}H)^r$ ..	$(11\text{-}H)^r$	H	A, B, C, D, E, F, G, J, K, L.	Nil	II	—
$(11\text{-}J)^r$ ..	$(11\text{-}J)^r$	J	A, B, C, D, E, F, G, H, K, L.	Nil	II	—
$(11\text{-}K)^r$ ..	$(11\text{-}K)^r$	K	A, B, C, D, E, F, G, H, J, L.	Nil	II	—
11^r . ..	11^r	Nil	All types	Nil	II	—

It will be seen from Table III that when a cholera vibrio was made resistant in the laboratory to all 'phage types except one and to all the 11 types of bacterio- phage, the resultant 11-1 resistant and 11 resistant strains, though derived from a typical agglutinable vibrio of the Ogawa type, fell into group II by inhibition test and the precipitin test with the sole exception of 11-A resistant. In order to ascertain whether a high degree of resistance to bacteriophage action *per se* was responsible for this grouping, a number of other strains, both agglutinable and inagglutinable, which on isolation were found to be highly resistant to bacteriophage, were tested (*vide* Table II). These results show that the acquisition of a high degree of bacteriophage resistance,. even to that of resistance to all types, when occurring in nature, does not necessarily bring about the reversion of an otherwise typical cholera vibrio from inhibition and precipitin group I to group II as it appeared to do when the resistance to bacteriophage action is artificially produced in the laboratory with the intervention of type A.

This appears to indicate that these tests depend not on bacteriophage resistance *per se*, but on some alteration in the constitution of the vibrio resulting therefrom, and that in the process of producing the resistant strains artificially with the intervention of type A the vibrios were apparently more profoundly altered than may be the case when the resistance is acquired naturally. In this connection one may compare strain F 5 (7) with W 127 and strain Etawah with U. P. V. Both the former strains are 11-1 resistant and the two latter strains are both 11 resistant, yet in each case one strain falls into group I and the other into group II.

Determination of the polysaccharide nature of vibrios by inhibition.—It was shown by Gough and Burnet (1934) that the 'phage inactivating agent present in the .extracts was a polysaccharide. They also showed that the polysaccharide of living bacterial cells obtained in their autolysates was more complex than that obtained in the usual haptene preparation.

In a previous communication (Pandit, Maitra and Datta Roy, *loc. cit.*), the inhi- bition groups, thought to be at the time three in number, were considered to corres- pond more or less to the grouping of Linton and Mitra (*loc. cit.*) according to the polysaccharides as determined by chemical analysis by these workers. Since the 'phage inactivating agent is considered to be of a polysaccharide nature, it was expected that a relationship could be established between this test and the actual polysaccharide of the vibrio. Among the organisms tested were twelve strains kindly sent by Dr. Linton, the nature of their polysaccharides having been deter- ·mined by him previously. These included strains representative of the various ' O ' sub-groups of Gardner and Venkatraman (1935) of which only one, i.e., Inaba, belonged to sub-group I. Inhibition tests were performed with extracts of these strains. The results are given in Table IV.

From Table IV it will be seen that only the Inaba strain fell into inhibition group I, the remainder falling into the heterogeneous group II. There was no relationship between the carbohydrate of the vibrio as determined by Linton and Mitra and the inhibition group to which it belonged. Inaba, which contained their carbohydrate I, fell into inhibition group I, but seven inagglutinable vibrios which also contained the identical carbohydrate, either by itself or in combination with carbohydrate III, all fell into inhibition group.II.

TABLE IV.

Extracts.	Strains.	Gardner–Bruce-White classification.	Action of the types of 'phage.		Types of 'phage inhibited.	Group by inhibition.	Actual polysaccharide by chemical analysis.
			Lysed by	Resistant to			
Inaba ..	Inaba	I	A, B, C, D, E, F, G, H, J, K, L.	*Nil*	A, B, D, F, H, J, K.	I	C I.
Inaba R ..	Inaba R	A	B, C, D, E, F, G, H, J, K, L.	A	*Nil*	II	C I.
N 32/124 ..	N 32/124	II	E, F, G, K	A, B, C, D, H, J.	E, H	II	C III.
N 32/77 ..	N 32/77	II	E	A, B, C, D, F, G, H, J, K, L.	B	II	C I.
N 32/77-R	N 32/77-R	B	E	A, B, C, D, F, G, H, J, K, L.	B	II	C I.
N 32/123 ..	N 32/123	III	*Nil*	All types	E, H	II	C III.
N 32/110 ..	N 32/110	IV	*Nil*	,,	*Nil*	II	C I and III.
N 32/110-R	N 32/110-R	C	K	A, B, C, D, E, F, G, H, J, L.	B	II	C I.
El Tor 34/D/19.	El Tor 34/D/19	V	B	A, C, D, E, F, G, H, J, K, L.	B, H	II	C III.
Kasauli 73	Kasauli 73	VI	F	A, B, C, D, E, G, H, J, K, L.	E	II	C III.
N 32/101 ..	N 32/101	Strain specific	D, J	A, B, C, E, F, G, H, K, L.	B, D, J	II	C I and III.
N 32/101-R	N 32/101-R	D	B	A, C, D, E, F, G, H, J, K, L.	B, H	II	C I.

SUMMARY.

1. By the test of inhibition of cholera-bacteriophage types by vibrio extracts prepared according to Burnet's method and by the precipitin reaction of these extracts, vibrios fell into two main groups. Group I contains typical smooth cholera vibrios, whilst group II, which is heterogeneous, contains water vibrios,

atypical cholera vibrios, including Inaba 'rough', the 'phage variants tested with the exception of 11-A and other non-agglutinating strains.

2. El Tor vibrios belonging to Gardner's 'O' sub-group I cannot be distinguished from typical smooth cholera vibrios by the inhibition and precipitin tests.

3. Both the inhibition and precipitin reaction appear to depend on a common factor which is related to the complex polysaccharide receptor of the cholera vibrio but bears no direct relation to the type or quality of polysaccharide by chemical analysis as found by Linton.

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AN INVESTIGATION OF VIBRIOS IN THE NETHERLANDS EAST-INDIES.

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IN the communications of the Public Health Office (D. V. G.) of 1937 a short reference was made to an investigation into the occurrence of vibrios in 20,444 samples of fæces and water, derived from non-cholera patients, from healthy persons, from some drinking water wells, pools and some other kinds of water, mentioned in the Annual Report Geneeskundig Laboratorium (1937).

The investigation was made at Dr. de Vogel's suggestion, who, seeing the great similarity between cholera and the El Tor strains and the uncertainty of the importance of the latter sort of vibrios, considered it of particular value to find out whether in these countries, where between 1927 and 1937 no cholera epidemics have been known to occur, this kind of vibrio is to be found either in man or in nature. In the course of this investigation 510 strains of vibrios were isolated, none of which possess the typical vibrio El Tor characteristics according to Shousha's indications. This investigation took place before the cholera epidemic at the end of 1937 on Celebes (de Moor, 1938). During the epidemic the El Tor vibrios were repeatedly found in the water from wells in the close vicinity of the cholera cases, as was stated by de Moor *(loc. cit.).* By the side of these waters vibrios were found, which according to Heiberg belong to the groups I and II and according to Gardner and Venkatraman to individual serological types.

The occurrence of agglutinable vibrios in healthy persons during a cholera epidemic has long been known. In an investigation of the fæces of 1,000 inhabitants of the Batavia kampongs during a cholera epidemic Flu has found from 6 to 7 per cent of carriers. The presence of inagglutinable vibrios was also stated by Flu (1914) in Batavia and by Mackie and Storer (1918) in Egypt and the same was found by Greig (1916) in an examination of water pools in the neighbourhood of Calcutta.

For many years the characteristic of micro-organisms of being strongly agglutinable with high-titre antisera (OH) has been used for the identification of *V. choleræ* but after the studies of Bruce White and others, who have shown the unspecific nature of the thermolabile (H) antigen of the vibrio, the 'O' antisera have been introduced into cholera diagnostics. Proceeding thus Gardner and Venkatraman (1935) have succeeded in grouping five 'O' types from a collection of vibrios not belonging to the classical cholera strains, by the side of a serologically entirely separate group of 'Individual' 'O' types, the antisera of which are only suitable to the homologous strain.

These investigators came also to the conclusion that the *V. choleræ* is a non-hæmolytic one with the specific 'O' antigen belonging to the sub-group I.

The investigations made by Taylor, Pandit and Read (1937) on 558 vibrios in-agglutinable with cholera 'O' sera, which had been isolated from the stools of cholera cases, from healthy carriers, and from water both in endemic and non-endemic regions, show that the above-mentioned serological types of vibrios are widely scattered all over British India.

The use of 33 antisera proved insufficient in British India to classify separately the 558 strains. Diversity, both as to biochemical qualities and to serological character, seems to occur more frequently among cholera-red negative cultures.

In analogy with the researches made in British India we have tried to identify the vibrios of various origin, found in this country, by exactly determining their biochemical and serological relations. For from an epidemiological point of view it is interesting to know whether the vibrio types occurring in British India are also found here.

As mentioned above, 510 vibrio strains were isolated from 20,444 samples of fæces and water. Thirty-two of them* reacted rather strongly to HO-serum from the Institute Pasteur at Bandoeng.

In our research we have restricted ourselves to the study of the 32 strains mentioned above, which, as regards their origin, may be divided thus :—

Fifteen strains from random patients (non-cholera cases) in the C. B. Z. (Central Clinics).

Sixteen strains isolated from stools of healthy persons, and one strain isolated from water.

As control matter we employed 10 cholera and 10 El Tor strains, which were sent to us by the Director of the Department of Public Health at Bangkok and by Dr. Doorenbos at Alexandria, to which gentlemen our heartfelt thanks are due, as also to Dr. de Moor who was kind enough to put at our disposal the Celebes strains isolated by him.

METHOD OF ISOLATION.

From the fæces inoculations were made in 30 c.c. of alkalized peptone water pH 7·8 and after 24 hours in the incubator sub-cultured on a Dieudonné plate. Of

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* Of these 29 originated from Java out of a total of 19,000, i.e., 0·15 per cent of fæces, samples examined.

One from Sumatra out of a total of 637 = 0·15 per cent of cases examined.

Two from the other islands out of a total of 804 = 0·25 per cent of cases examined.

the suspected colonies a preparation was made, stained by Gram's method, its motility checked in a hanging drop. When the motility of Gram-negative curved rods was high, the colony was more closely examined.

The isolation of vibrios from water was done as indicated by Kolle and Prigge (1928).

One litre of water sterilely drawn from well, river, or ditch was mixed with 100 c.c. concentrated peptone solution of the following composition :—

> 1 litre of aq. dest.
> 100 g. peptone Witte.
> 100 g. NaCl.
> 20 g. potassium carbonate.
> 1 g. potassium nitrate.

In addition 15 mg. of KCN per litre were added (Flu, 1915) to inhibit the growth of protozoa. This litre of water was divided over 10 tubes, which were placed in the incubator and after 6 and 24 hours sub-cultured in the usual fashion.

BIOCHEMICAL CHARACTERISTICS.

(a) In order to check the behaviour of the vibrios with regard to carbohydrates 1 per cent glucose, lactose, maltose, mannite and saccharose-peptone water are always used and the 'sugars' which Heiberg (1935) has used to differentiate the vibrios. Heiberg has found in his researches that the agglutinable vibrios belong to his group I, which statement has been confirmed by Taylor *et al.* (*loc. cit.*).

Gardner and Venkatraman (*loc. cit.*) divide the vibrios into three groups, viz., 'typical', 'atypical', and 'non-fermenting'. To the 'typical' group belong those strains which produce acid without gas in glucose, mannite, maltose and saccharose, give a positive cholera-red reaction, and do not ferment dulcite. In the 'atypical' group one or more of these biochemical qualities diverge from the normal character, whereas in the 'non-fermenting' group the carbohydrates are not acidified and gelatine is not liquefied.

From Table I, it appears that out of the 32 strains examined by us 10 belong to Heiberg's type I, 17 to type II, 3 to type III, and 2 to type IV. Furthermore, all our cholera and El Tor strains appear to belong biochemically to Heiberg's type I. So the type-differentiation after Heiberg shows us here too that the vibrios agglutinable with cholera-sera 'O' belong to group I. The reverse is, however, not the case, so that in diagnosing cholera vibrios we should not judge by fermentation reactions only.

(b) If we consider the results of the cholera-red reaction in several vibrio cultures isolated by us, it appears that in 10 of the fermentative type I, only 6 strains give a positive cholera-red reaction, whereas out of 17 of the fermentative type II, there are 14 with positive cholera-red. The three strains of type III, and two strains of type IV, all of them give a negative cholera-red. These results agree more or less with those of Taylor and his co-workers who found a positive cholera-red reaction in 90 per cent of the Heiberg's type I strains, a negative cholera-red in 342 cultures of type II, and a negative cholera-red in types III and IV.

According to Gardner and Venkatraman's division 20 of our strains, therefore, belong to the 'typical' group, 12 to the 'atypical' group and none to the 'non-fermenters'.

(c) In all our strains the indole reaction was positive, except in strain 8868, which belongs to the fermentation type Heiberg II.

(d) All strains liquefied gelatine.

(e) Voges-Proskauer reaction.—

In the carrying out of the V. P. reaction two methods were applied :—

1. The original reaction after Voges-Proskauer.—To 5 c.c. of three days' old 1 per cent peptone Witte-glucose culture are added 2 drops of a 2 per cent $FeCl_3$ solution and subsequently 5 c.c. of 10 per cent KOH solution. Kept for 2 hours at 37°C. and then at room temperature. The readings took place after 24 hours.

2. The modification after Barritt (1936), where KOH and α-naphthol cryst. med. (Schering Kahlbaum) are used is much more sensitive than the former. To 1 c.c. of 48 hours' old Difco-peptone culture are added 0·5 c.c. of KOH and 0·5 c.c. of α-naphthol alcoholic solution. The test may be read immediately and a positive reaction ·is to be recognized by the purple-red coloration.

The original V. P. reaction was only six times positive with our 32 strains. The modification after Barritt on the other hand gave many more positive results. Out of the 32 inagglutinable strains there are nine that gave no reaction. The results of the investigations of Taylor *et al.*, who assert that agglutinable non-hæmolytic vibrios should give a negative V. P. reaction after Barritt's modification, could not be confirmed by us with two Neth. Indian strains used. In Table III, we see that the cholera strains that had been inserted as controls in this experiment gave on the whole a negative V. P. reaction, an exception formed the strains V. C. Epidem. Nov. 1927 I and V. C. III (Mochtar and Baars, 1938).

The El Tor strains, however, gave a positive V. P. reaction after Barritt except D 84 and D 90.

(*f*) The hæmolytic capacity of the strains was examined as well in broth as in peptone cultures and on 5 per cent goat's blood agar plates.

In the examination of peptone cultures the hæmolysis test after Greig (*loc. cit.*) was applied : 1 c.c. of a 5 per cent saline suspension of once washed goat erythrocytes was added to an equal quantity of a 48 hours' old peptone culture. The original medium contained 1 per cent of peptone Witte with 0·85 per cent of NaCl whereas the pH was brought to 7·6. After the addition of the blood the tubes were shaken and for two hours kept in the incubator at 37°C. then transferred to cold storage until the following day. The reaction was read 24 hours after the addition of the blood.

For demonstrating the hæmolytic capacity of the strains in broth cultures the following technique was used :—

To isotonic Liebig broth fresh, defibrinated, and once washed goat's blood is added 24 hours before the inoculation. The inoculated tubes with the goat's blood at the bottom are incubated at 37°C. and daily checked as to the appearance

of hæmolysis. When looking at Table I, we see that out of the 32 strains 22 are distinctly hæmolytic according to both methods. As compared with Heiberg the results are as follows :—

Out of the 10 type I strains 7 are hæmolytic.
,, ,, ,, 17 ,, II ,, 14 ,, ,,
,, ,, ,, 3 ,, III ,, 1 is ,,
The 2 ,, IV ,, are not ,,

The application of the test after Greig has the following drawbacks :—

1. The presence of hæmotoxin is by this method shown only in 48-hour old cultures. The generally late appearance of endohæmolysin (Lampe) is not recognized by this method (which may be an advantage).

2. The exact moment at which hæmolysis sets in is thus not to be stated.

3. The duration of the action of the hæmotoxin is according to this method restricted to two hours.

Much more elegant we consider the method with blood-broth tubes, as recommended by van Loghem and described by Botman (1936). In his investigations about the variability of vibrios Botman has also made use of blood-broth cultures. Here the reading of the appearance of hæmolysis is indeed simpler, the hæmolytic capacity of each strain is separately and without titration to be compared. Hæmagglutination (which may make the tracing of hæmolysis difficult) is excluded. A drawback, however, is the insufficient contact of the hæmolysins with the goat-blood corpuscles, which may be intensified by shaking the blood corpuscles, which in van Loghem's method otherwise remain at the bottom of the tube of isotonic broth. All previous hæmolysis tests were made after van Loghem's method, so without shaking.

(*g*) The use of 5 per cent goat's blood agar plates yielded the following results :—

19 strains showed a combination of a green and a pale pink zone round the inoculating lines. On the pale pink zone a trace of oxyhæmoglobin is still to be discovered spectroscopically. With four strains an exclusive greening of the plate round the colonies is visible and with six strains the plates have remained unaltered.

Inoculation on blood-water plates showed a distinct greening (digestion) with 23 strains.

(*h*) The serological relations of the strains isolated were examined by testing the reaction with the ' H ' + ' O ' and ' O ' sera. The use of ' H ' + ' O ' serum was, as mentioned above, thought necessary for a tentative investigation. Thus, a large group of inagglutinable vibrios could be eliminated, which were immomentous for our purpose. This ' H ' + ' O ' serum was prepared from strains belonging to the ' O ' sub-group I after Gardner and Venkatraman.

It was more important to examine the behaviour of the isolated strains with regard to ' O ' sera. The ' O ' sera are obtained by injecting rabbits intravenously with a suspension of the agar-cultures that had been boiled for two hours. Besides ' O ' sera were also made from the dried antigens Inaba and Ogawa of Bruce White (especially prepared for routine work by the Standard Laboratory at Oxford).

These dried antigens were obtained by us through the kind intermediary of Dr. de Vogel and Colonel Taylor. With one mg. of this a rabbit was given three intravenous injections at intervals of five days. The end titres obtained of the different sera* are mentioned in the table. No serum of a titre lower than 1/3,200 was used for the test.

The bacterial suspensions are made by suspending a 24-hour old 3 per cent meat-agar culture in a physiological NaCl solution to which formalin has been added up to 0·25 per cent. The density of the suspension is always compared with the standard tubes (hæmocytometer method after Brown), and the density is chosen agreeing with the presence of 2 milliards of bacteria per c.c. of suspension. After the serum dilution and addition of the suspension the tubes are incubated at 37°C. Reading takes place the next day. Judging from the results obtained with the re-action to ' H ' + ' O ' Bandoeng serum of a titre 1 : 12,800 we noticed that three strains (280 BA, 1605, and 3488) reacted to a serum dilution of 1/6,400 (50 per cent), one strain (N : 4375) to a dilution of 1/3,200 (25 per cent), six strains, viz., 9952, 122, 2938, 3041, 418 BA, and 4152 to a titre of 1/1,600 (12·5 per cent), and the other 21 strains reacted only to a serum dilution of 1/200, 1/400 and 1/800.

If we consider the reaction to ' O ' sera (Inaba and Ogawa) it appears that there is no single strain that gives a high agglutination titre. Only the strains 280 BA, 1605, and 2938 give a secondary agglutination with a serum dilution of 1/400, with-out clarification occurring.

Thence the vibrio strains isolated by us completely deviate serologically from typical cholera vibrios.

In order to identify the serological types of our strains more accurately we have made agglutination tests with ' O ' sera, prepared from the British India strains 32/24, 32/23, 32/109, 34 D 19, and 73, respectively, belonging to the groups 2, 3, 4, 5, and 6 of Gardner and Venkatraman (kindly put at our disposal by Colonel Taylor) and also with ' O ' sera prepared from 12 of our own strains, viz., the strains 9952, 15/538, 122, 9049, 2938, 10774, 418, 4375, 3803, 7171, 306. and 2688.

Eleven out of the seventeen strains examined by the Central Research Institute at Kasauli (*cf.* last column of Table II) proved to agree serologically with vibrios found in British India in cholera cases.

The strain 2938 is serologically identical with the British Indian 8314 isolated from a carrier. The strain 3488 proved to be auto-agglutinable.

SUMMARY.

From the above we may deduce :—

1. That a number of vibrio strains, belonging to the types II, III, IV, and several so-called individual ones, after Gardner and Venkatraman, which in British India have been found in typical cholera cases and

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* In order to preserve the ' H ' + ' O ' sera phenol-glycerine is added in such a way that the phenol concentration comes up to about 0·25 per cent. A standard phenol-glycerine solution is started from the following composition : phenol 11, glycerine 40, physiological sodium chloride solution 200. Before use this solution is 10 × diluted and three parts of the diluted phenol solution are added to one part of serum. The thus treated sera keep longer in cold storage. The ' O ' antisera keep long when equal parts of glycerine are added to them.

have been isolated here from the fæces of healthy persons agree serologically. The results are contained in Table I. From this it appears that the use of 17 antisera is not sufficient to serologically classify the 32 strains. Most of the strains stand more or less by themselves so that there is no reason to assume serological unity. Two strains (2621 and 29 VIII$_{15}$) belong to type II of Gardner and Venkatraman, three strains (1605, 9205, and 10874) to type III, and three strains (3287, 280 BA, and 4152) to type IV. Only type V of Gardner and Venkatraman's is not represented in our collection.

2. That the anti-' O ' cholera sera yield specifically better results for diagnosis than cholera ' OH ' sera, if read after 24 hours and if clarification is assumed to be a criterium for ' O ' agglutination.

3. That the use only of the fermentative classification after Heiberg *is not satisfactory*, although all cholera strains were found by us to belong to type I Heiberg.

ACKNOWLEDGMENT.

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Biochemical and serological reactions of 32

Strains.	Date of isolation.	Source.	Motility.	Morphology.	Heiberg.	Indol.	Cholera-red.
9952 BA ..	4–8–36	Patient C. B. Z.	+	Gram-neg. vibrio	I	+	—
15/538 Dr. S.	4–5–36	,,	+	,,	I	+	—
122 Spec. ..	2–9–35	,,	+	,,	I	+	±
2621 ..	11–11–35	,,	+	,,	I	+	+
2688 ..	14–11–35	Healthy person	+	,	I	+	±
3016 ..	12–12–35	Patient C. B. Z.	+	,,	I	+	±
3803 ..	22–11–35	Healthy person	+	,,	I	+	+
7171 ..	8–6–36	Patient M. H.	+	,,	I	+	+
10299 ..	11–9–36	Healthy person	+	,,	I	+	—
10534 ..	25–9–36	,,	+	,,	I	+	+
9049 ..	5–8–36	,,	+	,,	II	+	+
280 BA* ..	2–3–36	Patient C. B. Z.	+	,,	II	+	+
1605 ..	30–7–36	Healthy person	+	,,	II	+	+
3488 ..	13–2–37	Patient C. B. Z.	+	,,	II	+	—
2686 ..	14–11–35	Healthy person	+	,,	II	+	+·
2687 ..	14–11–35	,,	+	,,	II	+	+
2938 ..	29–11–35	,,	+	,,	II	+	+
3041 ..	14–12–35	Patient C. B. Z.	+	,,	II	+	+·
7566 ..	25–6–36	Healthy person	+	,,	II	+	+
8868 ..	25–7–36	,,	+	,,	II	—	—
9205 ..	12–8–36	,,	+	,,	II	+	+
10021 ..	3–9–36	Patient M. H.	+	,,	II	+	+
10774 ..	14–10–36	Healthy person	+	,,	II	+	+
10874 ..	21–11–36	,,	+	,,	II	+	+
29 VIII$_{15}$ ..	7–4–36	Water	+	,,	II	+	—
418 BA ..	23–3–36	Patient C. B. Z.	+	,,	II	+	+
4375 ..	19–5–36	,,	+	,,	II	+	+
2962 ..	2–12–36	Healthy person	+	,,	III	+	—
3129 ..	2–1–36	,,	+	.,	III	+	—
3287 ..	11–1–36	Patient M. H.	+	,,	III	+	—
4152 ..	18–4–37	,, C. B. Z.	+	,,	IV	+	—
3203 ..	9–1–36	,, M. H.	+	,,	IV	+	—

* Strain 280 I belongs 100 per cent to

I.

non-cholera vibrio strains isolated in Java.

HÆMOLYSIS.		V. P. Barritt.	'H' + 'O' serum Bandoeng.	'O' SERA.			'O' SERA GARDNER AND VENKATRAMAN.			
Blood plate.	Blood bouil.			Inaba.	Hikojima.	Ogawa.	II 32/124.	III 32/123.	IV 32/109.	V 24 D 19.
··	−	+	1600	··	··	··	··	··	800	··
l	+	+	800	··	··	100	··	··	··	··
dl	+	+	1600	··	··	··	··	··	··	··
dl	+	±	400	··	··	··	3200	··	··	··
dl	+	−	800	··	··	··	··	··	··	··
dl	+	+	400	··	··	100	··	··	··	··
dl	+	+	200	200	··	··	··	··	··	··
d	−	+	400	··	··	100	··	··	··	··
dl	+	+	400	··	··	100	··	··	800	··
d	−−	+	400	··	··	··	··	··	··	··
dl	+	+	400	··	··	100	··	··	··	··
dl	+	+	6400	200	··	400	··	··	6400	··
l	+	+	6400	400	··	400	400	3200	··	··
l	+	−	6400	200	··	100	··	200	··	··
dl	+	+	800	··	··	··	··	··	··	··
dl	+	+	800	··	··	··	··	··	··	··
dl	+	+	1600	400	··	100	··	··	··	·
dl	+	+	1600	200	··	··	··	··	··	··
dl	+	+	400	200	··	··	··	··	··	··
··	−	−	200	200	··	··	··	··	··	··
dl	+	−	200	··	··	··	··	6400	·	··
dl	+	+	200	··	··	··	··	··	··	··
dl	+	+	400	··	··	··	··	··	··	··
dl	+	+	400	··	··	··	··	6400	··	··
··	−	+	200	··	··	··	3200	··	··	··
dl	+	+	1600	··	··	··	··	··	··	··
d	−	+	3200	··	··	··	··	··	··	··
dl	+	−	400	··	··	··	··	··	··	··
··	−	−	400	200	··	200	··	··	··	··
d	−	−	200	··	··	100	··	··	6400	··
··	−	−	1600	200	··	··	··	··	6400	··
··	−	−	400	··	··	100	··	··	400	··

type VI of Gardner and Venkatraman.

TABLE II.

Serological reactions of Java non-cholera strains including relationship to Indian non-cholera strains.

Strains.	ANTISERA.																	Serology with standard 'O' sera from Central Research Institute, Kasauli.
	9952	15/538	122	9049	2938	10774	418	4375	2688	3016	3803	7171	G 2 32/124	G 3 32/123	G 4 32/109	G 5 34/D 19	G 6 73	
9952 BA	6400	3200																..
15/538	3200	3200																..
10299	6400	6400																..
122 Spec.			6400															100 per cent 627/29/2 (Calcutta case strain).
10554			6400															..
9049				6400														100 per cent 324/7 (Calcutta case strain).
10021				6400														..
2938					6400													100 per cent 8814 (Calcutta carrier strain).
10774						6400												100 per cent 1612 (Calcutta case strain).
418 BA							6400											20 per cent Rangoon 'rough' (Rangoon case strain).
4375								6400										100 per cent 324/7 (Calcutta case strain).
2688									6400									*Nil.*

No.									Remarks
3016	..	..	..	..	..	..	..	6400	*Nil.*
3803	..	..	..	..	..	..	6400	..	
7171	..	..	..	..	..	6400	..	..	100 per cent G II (Nanking case strain).
2621	..	..	..	..	3200	..	..	..	
29 VIII₁₀	..	..	..	3200	3200	..	..	..	100 per cent G III (Nanking case strain).
1605	..	..	3200	..	..	..	..	..	100 per cent G III (Nanking case strain).
9205	..	..	6400	..	..	..	..	..	100 per cent G III (Nanking case strain).
10874	..	..	6400	..	..	..	..	..	100 per cent G III (Nanking case strain).
280 BA	6400	..	..	..	..	..	..	..	
3287	6400	..	..	..	..	..	..	..	
4152	6400	..	..	..	..	..	..	..	*Nil.*
2687	..	..	..	..	..	..	..	..	
8868	..	..	..	..	..	..	..	..	100 per cent L/3/36 (Lahore case strain).
3203	..	..	..	..	..	..	..	..	
2962	..	..	..	..	..	..	..	..	*Nil.*
3129	..	..	..	..	..	..	..	..	Auto-agglutination.
3488	..	..	..	..	..	..	..	..	
2686	..	..	..	..	..	..	..	..	
3041	..	..	..	..	..	..	..	..	
7566	..	..	..	..	..	..	..	..	

TABLE III.

Biochemical and serological reactions of cholera and El Tor strains for comparison.

Number of the strains.	Date of isolation.	Origin.	Motility.	Morphology.	Saccharose.	Arabinose.	Mannose.	Type Heiberg.	Indol.	Cholera-red reaction.	Haemolysis.	V. P. Werkman.	V. P. Barritt.	'H', '+', 'O' serum Bandoeng 12000.	Inaba 6400.	Ogawa 6400.
															'O' SERA.	
Cholera:																
V.C. 91/79 ..	April 1936	Bangkok	+	Gram-neg. vibrio	+	−	+	I	+	+	−	−	−	6400	3200	3200
V.C. 137/79 ..	May 1936	,,	+	,,	+	−	+	I	+	+	−	−	−	6400	6400	6400
V.C. 153/79 ..	,,	,,	+	,,	+	−	+	I	+	+	−	−	−	6400	3200	3200
V.C. 155/79 ..	,,	,,	+	,,	+	−	+	I	+	+	−	−	−	6400	6400	3200
V.C. 157/79 ..	,,	,,	+	,,	+	−	+	I	+	+	−	−	−	6400	6400	6400
V.C. 301/79 ..	September 1936	,,	+	,,	+	−	+	I	+	+	−	−	−	6400	6400	3200
V.C. 302/79 ..	July 1937	,,	+	,,	+	−	+	I	+	+	−	−	−	6400	3200	3200
V.C. 304/79 ..	,,	,,	+	,,	+	−	+	I	+	+	−	−	−	6400	6400	3200
200/a 1 ..	,,	,,	+	,,	+	−	+	I	+	+	−	−	−	6400	3200	3200

	Cl. Ep. Nov. I	D 82	D 83	D 84	D 85	D 86	D 87	D 88	D 89	D 90	D 91
	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200	1600
	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200	3200
	6400	6400	6400	3200	6400	6400	6400	6400	6400	6400	6400
	−	+	+	−	+	+	+	+	+	−	+
	−	−	+	−	+	+	−	−	−	−	+
	−	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+
	H	H	H	H	H	H	H	H	H	H	H
	+	+	+	+	+	+	+	+	+	+	+
	−	−	−	−	−	−	−	−	−	−	−
	+	+	+	+	+	+	+	+	+ +	+	+
	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
	+	+	+	+	+	+	+	+	+	+	+
	,,	El Tor	,,	,,	,,	,,	,,	,,	,,	,,	,,
	,,	1937	,,		,,	,,	,,	,,	,,	,,	,,
	..	..	..	..	..	..	..	..	..	..	..

THE NUTRITIVE VALUE OF PAPAIN-MUTTON BROTH.

BY

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THE degree to which papain carries the digestion of protein has been investigated by a number of workers. Wurtz (1880) found leucine to be amongst the products of papain digestion. Chittenden, Mendel and McDermott (1898), however, showed that the chief products of papain digestion were deutero-albumose and peptone. These investigators showed that even under the most favourable conditions the amino-acid formation was very slight. In 1901 Vines, on the other hand, reported positive tryptophane tests with chlorine water in papain digestion of fibrin. But Mendel and Underhill (1901) in over sixty digestion experiments, using four different samples of papain and casein, fibrin, coagulated egg-white, and boiled and raw muscle as substrates, obtained evidence of leucine, tyrosine, and tryptophane with uncoagulated muscle only. This exception they attributed to autolytic action. Mendel (1902) pointed out that the results of Vines were open to the objection that no antiseptic was used and the possibility of the bacterial origin of the tryptophane was not excluded. Modern researches, however, have cleared the question as to the extent of the hydrolysis by papain. It has been found that papain digests protein only up to the peptone stage; thus peptones, whether obtained by peptic or by papain digestion, are not attacked by papain but are split only in the presence of hydrocyanic acid (Waldschmidt-Leitz, 1929). This activation, first recognized by Mendel and Blood (1910), has been extensively investigated by Frankel (1917). Thus, although papain is a true protease, it becomes capable of attacking peptone only when activated by hydrocyanic acid. This point is stressed to show that in the process of preparation of papain-mutton digest medium for the cultivation of micro-organisms there is little possibility of free amino-acid being formed.

Martin (1927) first made use of papain in the preparation of mutton broth for bacteriological purposes. Martin's method consisted in digesting mutton with papain for six hours at temperatures varying from 50°C. to 80°C. The papain-mutton broth thus prepared has since been found to be one of the best and cheapest media for general use. A perusal of the literature shows that no work has been done on this subject to determine the exact period of complete digestion of mutton with papain. Asheshov, Asheshov, Khan and Lahiri (1933) state that papain-digested meat media prepared by carrying out digestion at 70°C. for three hours was quite

suitable for the growth of *V. choleræ* and its bacteriophage. Recently, Vardon and Datta Roy (1938) prepared a papain-casein culture medium and found that at 70°C. to 80°C. 1·5 grammes of papain digested one litre of 2·5 per cent casein solution in approximately two hours.

The present investigation has been for the purpose of determining the period of complete digestion of mutton with papain in the preparation of papain-mutton broth and for ascertaining the products at different stages of digestion in relation to the yield of vibrio growth.

Experimental.

The progress of digestion of mutton with papain was followed by determining the amino-nitrogen, oxidizable matter, and total nitrogen. Proteins, proteoses, peptones, and residual nitrogen were also determined at various stages of digestion. Finally, the yield of *Vibrio choleræ* grown on papain-mutton agar slopes prepared from the digest obtained at different stages of digestion was estimated. The biuret test, as it is generally done to follow the course of digestion, has not been found to indicate the stage of digestion reached. Thus, the biuret test showed a rose-red coloration when digestion progressed only for half an hour but by chemical analysis it was found that much undigested protein was still in solution at that period.

Digestion of mutton with papain.

The digestion was carried on for half, one, two, three, four, five, and six hours, respectively, in separate flasks. The mixture in each flask was prepared as follows :—

Three hundred grammes of minced mutton free from fat was accurately weighed and rubbed up with 5 grammes of powdered papain in a mortar. This was then stirred with 200 c.c. of distilled water and transferred to a flask. A litre of distilled water was then added, thereby making the total volume of water to 1,200 c.c. The flasks, each containing the above mixtures, were then placed in a water-bath maintained at 60°C. The reactions were adjusted to pH 7·0 and re-adjusted after every hour by the addition of 10N alkali. Two control experiments were also set up without the addition of papain and estimations made immediately on preparation and after 6 hours in the water-bath. After the requisite periods of digestion, the flasks were taken out of the water-bath and strained through muslin. The turbid filtrates were then autoclaved at 15 lb. pressure for half an hour to inactivate the enzyme. When cold the contents of each flask were filtered off from its coagulum. One hundred cubic centimetres were taken from each of the clear filtrates thus obtained for chemical analysis. The rest of the filtrates were used for preparing the nutrient agar.

Chemical analysis of the broth.

1. *Determination of amino-nitrogen.*—The formol titration first developed by Sørensen (1907) for determining amino-nitrogen has been modified by different workers. Brown (1923) modified the formol titration for determining amino-nitrogen in bacteriological media. The method adopted in the present investigation was essentially that of Brown and the results at different stages of digestion are

given in Table I. It was found that the formol titration figure increased as the digestion progressed and came to a maximum at the end of two hours, after which it remained constant. This shows that after two hours of digestion, there is no further appreciable liberation of amino-nitrogen.

2. *Determination of oxidizable matter.*—The determination of oxidizable matter by the permanganate process has been found to give a very fair estimation of the nutrient properties of the medium. The method has been described in detail by Asheshov *et al. (loc. cit.).* The percentages of oxidizable matter at different periods of digestion are shown in Table I. It was found that the oxidizable matter increased with the course of digestion and like the formol titration figure reached a maximum at the end of two hours, after which it remained constant.

3. *Determination of total nitrogen.*—The nitrogen was determined by Kjeldahl's process and the amounts at different stages are recorded in Table I. It was found that like the results found by the formol titration and oxidizable matter estimation the total nitrogen content increased as the digestion progressed and became maximum at the end of two hours, after which it remained constant.

The above three estimations, i.e., by formol titration, estimation of oxidizable matter and total nitrogen, all show that the digestion of mutton with papain at 60°C. is complete at the end of two hours. The course of digestion can, therefore, be followed by any of the above three methods, but of these, the formol titration has been found to be the simplest and is recommended for determining the completeness of digestion of papain-broth.

The figures for amino-nitrogen, oxidizable matter, and total nitrogen of the two controls before placing in the water-bath and after six hours at 60°C. are shown in Table I for comparison :—

TABLE I.

Broth.	Formol titration, mg. of amino-nitrogen per 100 c.c.	Percentage of oxidizable matter.	Total nitrogen, mg. per 100 c.c.
0 hour	14	0·2	92·4
½ ,, ...	42	0·5	204·4
1 ,, ...	84	1·0	422·8
2 hours	140	1·5	587·7
3 ,, ...	140	1·5	588
4 ,, ...	140	1·5	588
5 ,, ...	140	1·5	588
6 ,, ...	140	1·5	590
0 hour control (without papain).	3·5	0·05	25
6 hours control (without papain).	7·0	0·10	58

The experiments were repeated with half the quantity of papain and the amino-nitrogen, oxidizable matter and total nitrogen estimated as before at different periods of digestion. The results are recorded in Table II. It will be seen that the digestion of 300 g. of mutton with 2·5 g. of papain was complete at the end of 3 hours instead of 2 hours found in the previous experiment where double the quantity of papain was used. The formol titration and oxidizable matter figures reached the same maximum level in both the digestion experiments. The maximum total nitrogen figure obtained in the present experiment was, however, about 18 mg. less than that found in the previous one. This is shown to be accounted for by the lesser quantity of papain used in this experiment; as in a control experiment it was found that 2·5 g. of powdered papain itself under the above experimental conditions contributes about 22 mg. of nitrogen from 2 hours onwards.

TABLE II.

Broth.	Formol titration, mg. of amino-nitrogen per 100 c.c.	Percentage of oxidizable matter.	Total nitrogen, mg. per 100 c.c.
0 hour ..	14	0·25	94
½ ,, ..	35	0·45	201
1 ,, ..	63	0·80	326
2 hours ..	112	1·1	394
3 ,, ..	140	1·5	570
4 ,, . ..	140	1·5	572
5 ,, ..	140	1·5	571
6 ,, ..	140	1·5	571

4. *Estimation of proteins, proteoses, peptones, and residual nitrogen.*—Wasteney's and Borsook's (1924) method was followed in this investigation for the quantitative estimations of these fractions. Briefly the method was as follows:—

The protein was precipitated by the addition of 10 per cent trichlor-acetic acid. The final concentration of this reagent did not exceed 2 per cent. Proteoses were precipitated from the above filtrate at 33°C. by the addition of excess of anhydrous sodium sulphate. Peptones were estimated from the sodium-sulphate filtrate by precipitation with tannic acid. The nitrogenous material remaining in the filtrate from the tannic acid precipitate, consisted of sub-peptones, amino-acids, and amides. Proteins, proteoses, peptones, and residual nitrogen were all estimated by their total nitrogen content, obtained by difference before and after filtration.

TABLE III.

Broth.	Total nitrogen, mg. per 100 c.c.	Percentage of protein nitrogen.	Percentage of papain nitrogen.	Percentage of proteose nitrogen.	Percentage of peptone nitrogen.	Percentage of residual nitrogen.
0 hour ..	92·4	43·8	5·4	34·8	18·2	3·2
½ ,, ..	204·4	28·2	12·2	41·8	24·8	5·2
1 ,, ..	422·8	9·9	7·8	62·3	20·7	7·1
2 hours ..	587·7	6·4	7·6	48·7	37·4	7·5
3 ,, ..	588	7·0	7·6	44·2	38·6	10·2
4 ,, ..	588	7·0	7·6	40·3	40·8	11·9
5 ,, ..	588	6·5	7·6	40·0	41·6	11·9
6 ,, ..	590	6·8	7·6	39·0	42·4	11·8

The percentage content of protein nitrogen, proteose nitrogen, peptone nitrogen, and residual nitrogen, at different periods of digestion are recorded in Table III. A control experiment with papain only was also performed and the percentages of papain nitrogen at different stages are given in the same table. The papain-protein content could not be estimated owing to the auto-digestion of papain. It will be seen from the table that the percentage of protein nitrogen of mutton decreases as the digestion progresses and comes to a low but constant figure at the end of 2 hours. This amount of protein nitrogen from the second hour onwards is fully accounted for by the amount of papain nitrogen contained at those periods. It will be noticed that the papain-nitrogen figures from the second hour onward are slightly in excess of that of protein nitrogen of mutton at those periods. This is probably due to the presence of non-protein nitrogenous matter contained in papain. The transformation of mutton protein is therefore complete at the end of 2 hours. In the course of the examination the phenomenon of sudden digestion by papain acting on animal tissues was noticed. In samples examined immediately on preparation of papain-mutton mixtures it was found that 50 per cent of the nitrogen were protein split products. This phenomenon of sudden digestion by papain has not been noted in the case of the action of other enzymes and has been reported by Pozerski (1909). He found that 30 per cent of albumin was digested when papain had been left one minute in direct contact with the albumin at 18°C. and then rapidly brought to 100°C. to destroy the enzyme activity. He stated that the duration of heating which had been from 10 to 20 seconds was quite sufficient to bring about this remarkable effect. In this present investigation the immediate digestion of mutton did not occur as long as the temperature was low because the hydrolysis with papain begins in the neighbourhood of 50°C. The digestion, on the contrary, took place suddenly between 60°C. and 90°C. during 1 or 2 minutes in sterilization with a view to killing the enzyme for the purpose of analysis at that stage. With regard to the proteose-nitrogen content, it will be noticed that the percentage of proteose nitrogen increased as the digestion progressed and reached its maximum at the end of

1 hour. From 1 hour onwards the proteose-nitrogen content decreased. The percentage of peptone nitrogen, on the other hand, increased all throughout the course of digestion. The percentage of residual nitrogen increased with the digestion and remained constant from the fourth hour onwards. The tests for tryptophane and tyrosine were found to be negative, hence this residual nitrogen fraction represents only the sub-peptones and amides.

We have seen that as estimated by formol titration, oxidizable matter, and total nitrogen content, a constant figure is obtained at the end of 2 hours of digestion. This is also the period when the transformation of protein is complete. The only change that takes place after 2 hours is the slow transformation of proteose to peptone. The formol titration method was, however, unable to detect such slow change during 2 to 6 hours of digestion.

Growth of *Vibrio cholerœ.*

Nutrient agar, prepared from papain digests at different periods by the addition of 0·5 per cent sodium chloride and 4 per cent agar, was placed in roll bottles and sterilized. The bottles were then sown with a heavy inoculum of *V. cholerœ.* The bottles were incubated for 24 hours and the growth was thoroughly washed off with normal saline. The yield of cholera vibrios was then estimated by Brown's opacity tubes and the results are given in Table IV. It will be noticed that the yield was at its maximum in digests at 2 hours and did not increase when digests of longer period were used. This corresponds with the results of chemical analysis.

Table IV.

Nutrient agar.	Yield of cholera vibrio per c.c.
0 hour ..	$32,780 \times 10^6$
½ ,, ..	$49,170 \times 10^6$
1 ,, ..	$57,360 \times 10^6$
2 hours ..	$65,550 \times 10^6$
3 ,, ..	$65,550 \times 10^6$
4 ,, ..	$65,550 \times 10^6$
5 ,, ..	$65,550 \times 10^6$
6 ,, ..	$65,550 \times 10^6$

Summary.

The period of digestion of 300 g. of mutton mince with 5 grammes of powdered papain as estimated by formol titration, oxidizable matter content, and total nitrogen content, has been found to be complete after 2 hours at 60°C. With half

the quantity of papain the digestion was delayed by 1 hour. Of the above three estimations used to follow the course of digestion, the formol titration has been found to be the simplest. The transformation of protein has been found to be complete after 2 hours of digestion. The only change that took place after that period was the slow transformation of proteose to peptone. The growth of the cholera vibrio on papain-mutton agar at different stages of digestion showed that the maximum growth was obtained after 2 hours of digestion which was also the period of complete digestion as determined by chemical analysis.

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NOTE ON THE PREPARATION OF AN UNPURIFIED MANNOSE SOLUTION FOR BACTERIOLOGICAL USE.

BY

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(Cholera Inquiry under the I. R. F. A., Central Research Institute, Kasauli.)

IN view of the value of mannose, which is an expensive sugar, as a carbohydrate for use in classification of vibrios by fermentation methods and the extensive use which has been made in this Institute in differential isolation of vibrio types (*vide* Read, 1939), the preparation of an unpurified solution has been undertaken, as a matter of economy. For the purpose, ivory-nut shavings which contain a high percentage of mannose have been used. A supply of these, which have little commercial value and are the waste products of button manufacture, was obtained from the Rochester Button Co., Rochester, New York, and the method of extraction employed was that described by Horton (1921) and by Clark (1922). The preparation of this product from ivory-nut shavings was carried out as follows :—

Two hundred grammes of ivory-nut shavings were added to two litres of boiling 1 per cent sodium hydroxide solution. The mixture was at once removed from the source of heat and stirred frequently during half an hour. The extract was filtered off and the residue washed with tap water until the filtrate was clear and neutral. The extracted residue was then dried in a water-jacketed oven at a temperature below 60°C. The dried extracted meal thus prepared was thoroughly mixed with 110 c.c. of cold, 65 per cent by weight. sulphuric acid and allowed to stand overnight. This mass was taken up with 1,200 c.c. of distilled water and boiled under a reflux condenser on a sand-bath for two and a half hours. The hot liquid was neutralized by adding slightly less than the calculated amount of powdered barium hydroxide. The neutralization was finally completed with powdered barium carbonate and the solution at once filtered. The filtrate contained a little barium because of the solubility of barium carbonate and also because of the combination of barium with organic acids. The excess of barium was removed by adding carefully dilute sulphuric acid until no further precipitate of barium sulphate was formed. The barium sulphate was filtered off. The filtrate obtained was light yellow in colour. Mannose was then directly estimated in an aliquot of this final filtrate by the formation of

mannose phenylhydrazone. The weight of the dried phenylhydrazone multiplied by the factor 0·666 gave the weight of mannose. The percentage of mannose in the solution was found to be 8·50.

Three pounds (i.e., 1,360·77 g.) of ivory-nut shavings altogether were treated in the above way and the amount of mannose in the final product was found to be 620·0 g. The yield of mannose was, therefore, 45·56 per cent.

The cost of production from the shavings supplied free was that of a small amount of caustic soda, some sulphuric acid and barium hydroxide, and carbonate, a total approximating Rs. 3.

The fermentation of the unpurified product was tested with known mannose-fermenting and mannose-non-fermenting vibrios and the results conformed with those obtained when the pure carbohydrate was employed. In the method of differential isolation described by Read (*loc. cit.*) the substitution of the unpurified product for the pure sugar gave equal results.

SUMMARY.

The application of a recognized method for the preparation of an unpurified solution of mannose from vegetable ivory-nut shavings has been found to yield a cheap product which can be substituted for the use of pure mannose in bacteriological work in connection with vibrios.

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THE NUTRITIONAL REQUIREMENTS OF THE PLAGUE BACILLUS.

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INTRODUCTION.

THE study of the nutritional requirements of bacteria has a twofold importance. Firstly, it throws light on the more obscure synthetic or growth phases of bacterial metabolism, and secondly, it offers the only sound basis possible for designing special nutritionally complete media. As an example of the latter may be cited a protein-free 'synthetic' or chemically defined medium which possesses considerable advantages over the more complex laboratory media for the isolation of antigens or toxins from cultures of pathogenic bacteria. In the present paper the nutritional requirements of the plague bacillus, *Pasteurella pestis,* are described with the hope that it will not only serve the needs of those engaged in immunological or bacteriological work but will also serve as a starting point for the further study of its metabolism.

Recent investigations in bacterial nutrition, and particularly those of Fildes and his co-workers, have helped to co-ordinate the divergent observations recorded previously. Since the present work is on identical lines the special technique as well as the generalizations developed by these workers are briefly summarized here. The literature on the subject has been reviewed by Knight (1936). He presents all the types of bacterial nutrition as an evolutionary series. Thus, the autotrophs which possess a pronounced ability for assimilating CO_2, are on a lower level than the heterotrophs which are dependent on ready elaborated carbon compounds. The powers of assimilating nitrogen provide a series of greater significance. The most primitive types synthesize all their body proteins out of inorganic source of nitrogen (N_2, NH_3 or NO_3). At a higher level amino-acids are necessary, in particular amino-acids of complex structure such as tryptophane. Finally, at the highest level, wherein belong most pathogens, not only are a series of different amino-acids necessary, but also certain accessory growth factors or 'bacterial vitamins' (Kögl *et al.,* 1937 ; Lwoff, 1938). Knight draws the important conclusion that an increase in nutritional complexity is equivalent to a decrease *through loss* of synthetic powers, and is in general correlated with parasitism. From this emerges the definition of a growth factor, viz., a chemical molecule of a definite configuration

which a given micro-organism is incapable of synthesizing for itself out of simpler compounds, and in the absence of which growth and multiplication are impossible. It is convenient to exclude inorganic elements, Fe, Mg, Cu, etc., from this definition, but include only amino-acids and accessory factors. The investigation of nutrition, therefore, amounts to the determination, under conditions rigorously excluding the presence of all other unknown compounds, the smallest number of growth factors required to maintain growth. With bacteria of complex nutrition the most direct method, as was first employed by Knight and Fildes (1933), is to employ a basal deficient medium of known chemical composition, such as hydrolysed gelatin, and test its growth supporting ability after adding other amino-acids and accessory factors in the shape of fractions from natural sources. The conclusions indicated are confirmed by using synthetic amino-acids and purified or synthetic accessory factors. The detailed procedure is described by Fildes and Richardson (1935, 1937), Gladstone (1937), Knight (1937*a*, *b*), and Richardson (1936). Similar work has been done by Mueller on the diphtheria bacillus (Mueller, Klise, Porter and Graybiel, 1933, and later papers).

In the present study of the nutritional requirements of the plague bacillus attention has been directed towards answering the following fundamental questions :—

1. Can the plague bacillus be grown in a simple chemically defined medium ?
2. What are the accessory factors involved in its nutrition ?
3. Are there significant nutritional differences between the different strains, and between the virulent and the avirulent strains ?
4. What are the amino-acids indispensable as growth factors ?

TECHNIQUE.

Bacterial strains.—The following plague strains, from the collection at this Institute, were used in the nutrition experiments :—

Virulent strains : 36H, 37H, 38B, 39B, 120/5H, and I.

Avirulent strains : 120/5H, I, P, and Q.

The routine method of maintaining the virulent strains (Sokhey, 1935, 1936) throws light on their nutritional history. From the primary culture from the patient's blood (from septicæmic cases of recent epidemics) after testing for purity, a large number of sub-cultures had been made on rabbit-blood agar in test-tubes, which were then sealed and stored at 4°C. ± 2. The virulence was fully maintained and was being controlled in connection with other investigations. There was little chance, therefore, for nutritional adaptation to have occurred after isolation. The avirulent strains had been developed from the virulent ones of identical names in the course of other work (Sokhey, 1936 ; Sokhey and Maurice, 1937) by repeated sub-culturing, and had been similarly stored. The avirulent strains, therefore, had a chance of undergoing considerable adaptation in nutrition.

The basal medium.—Hydrolysed gelatin was prepared according to the method of Knight and Fildes (*loc. cit.*) from Merck's Gold Label brand gelatin, and used in 0·5 per cent concentration (containing about 0·9 mg. total N per c.c.) as the basal medium in experiments described under Section I below. In experiments detailed under Section II, the basal medium, after adding cystine, was adjusted to contain

2·3 mg. total N per c.c. corresponding to that of the acid-digest broth used in this Laboratory for growing the organism.

Sources of accessory factors.—To answer the question whether accessory factors were involved in nutrition, the following natural sources likely to be rich in such factors were used in 1×10^{-4} concentration : (1) Marmite (autolysed yeast), (2) meat extract, (3) mare's urine, and (4) heated rabbit blood. Further fractionation of these was not found necessary since, as will be shown below, they supplied no accessory factors whatever for the growth of the plague bacillus.

Amino-acids.—The results obtained with hydrolysed gelatin were confirmed and extended by using media made with pure amino-acids (Sections III, IV, and V). The special precautions to be taken in such experiments to secure entirely reliable results have been stressed by Fildes and Richardson (1935). Only synthetic amino-acids must be used to obviate interference from traces of other amino-acids which are likely to be present as impurities in natural amino-acids. The effective concentrations of these compounds in bacterial nutrition have been found to have magnitudes extremely small to be detected by chemical tests, for example 10^{-5} M. to 10^{-6} M. arginine in the growth of *Clostridium sporogenes* (*loc. cit.*). In fact controlled bacterial growth tests appear to be the most sensitive for such compounds. Accessory factors act as a rule at even lower concentrations, for example, phosphopyridine nucleotides are active at a concentration of 6×10^{-8} in the growth of *Hæmophilus influenzæ* according to Lwoff and Lwoff (1937). The difficulty is best overcome by using synthetic amino-acids as far as possible, and by conducting the tests at low ranges of concentrations where interference from impurities is reduced to a minimum. In the experiments described in the present paper the following synthetic amino-acids were employed : *dl*-alanine, *dl*-leucine, *dl*-proline, *dl*-phenyl-alanine, *dl*-cystine (Schuchardt), and glycine (Merck). Owing to the fact that corresponding synthetic compounds were not available *d*-lysine 2HCl and *d*-arginine (Schuchardt) from natural sources were used. Natural *l*-cystine and *l*-tryptophane (Merck) were also employed in the experiments under Section I. The concentrations of the amino-acids in the media used are given in Table II, and have been calculated for one isomer only in the case of racemic acids.

Inorganic requirements.—Inorganic needs were supplied by adding to all the media a salt and buffer mixture. This consisted of a M/15 phosphate buffer at pH 7·0, with the addition of sodium citrate (cryst) 4·0 g. and magnesium sulphate (dry) 0·5 g. per litre.

Preparation of media.—Growth tests were conducted in thick-walled hard glass test-tubes of uniform diameters, which were cleaned first with concentrated chromic-sulphuric acid mixture. Into each test-tube were added 5 c.c. salt and buffer mixture, the other constituents and distilled water being then added to make up to 10 c.c. final volume. It was found more convenient to mix the constituents before sterilization, though the alternate method of mixing after sterilizing the constituents in bulk gave identical results. Sterilization of all media was done under steam at 128°C. for 20 minutes.

Inoculation.—In order that growth factors or other unknown compounds should not be introduced into the medium with the inoculum, thoroughly washed bacterial cells only were used. Preliminary experiments showed that an inoculum

of washed plague bacilli must be of a sufficiently large size before it can grow in nutrient broth. Thus, three inocula of 7×10^5, 7×10^4, and 7×10^3 numbers of washed bacteria were sown into three test-tubes of 10 c.c. broth respectively. The first tube gave visible growth in 24 hours and the second in 48 hours, while in the third the inoculum failed to grow. The cause of the failure must be sought among factors of the experiment other than growth factors, possibly in oxidation-reduction potentials. The difficulty was, however, easily overcome by using fairly large inocula of bacterial suspensions which had been thoroughly washed on the centrifuge to remove all possible traces of adhering metabolities. The procedure was as follows : The sealed stock sub-culture was opened and a colony or two of bacteria were emulsi-fied into 10 c.c. of acid-digest broth in a test-tube, which was then incubated for 48 hours at 27°C. The broth culture was then planted on agar slopes (acid-digest broth) and grown for another 48 hours at the same temperature. Bacteria which are grown at a higher temperature, for example 37°C., cannot be centrifuged satis-factorily. The growth was washed out in sterile saline without scraping the surface and the suspension was centrifuged at 5,000 r.p.m. for 10 minutes. The supernatant fluid was then poured off and fresh saline introduced and the bacteria thoroughly stirred up with a fresh pipette. The washing and centrifuging were repeated five times, with precautions against atmospheric contamination at each stage. The suspension was finally adjusted to an opacity No. 1 on Brown's scale and exactly 0·2 c.c. was inoculated into each experimental media tube. This inoculum, while large enough to give successful growth in 24 to 48 hours, did not confer detectable turbidity to the medium.

Growth observations.—The tubes were incubated at the optimum temperature for growth, 27°C. (Sokhey, 1937), and were examined at intervals of 24 hours. The turbidity due to growth was noted and recorded with the following set of relative values :—

     0 = no growth, the medium remains crystal clear.

  tr. = traces of growth.

   + = distinct growth.

++ = growth corresponding to opacity No. 1.

Since the crucial test with any medium is whether it supports the growth of the bacillus or not, quantitative estimation of growth is not necessary. According to the usual criteria, the entire absence of growth was taken to indicate the absence of one or more growth factors, while its appearance in 24 to 48 hours indicated the presence of all the growth factors. Delayed growth was taken to mean the absence of an important, but not indispensable, factor. As a final proof that the organism found growing was the bacillus under experiment and not a contaminant, routine diagnostic fermentation tests and other purity tests were made with tubes taken at random at the close of each experiment. In no case was any organism other than the plague bacillus found.

EXPERIMENTAL RESULTS.

I. *A preliminary survey.*—The experiments of this series were planned to obtain a general idea of the amino-acids involved in the nutrition of the plague bacillus and the sources in which accessory growth factors, if any, occur. Hydro-

lysed gelatin (0·5 per cent) was taken as the basal medium, and its growth-promoting ability was tested alone as well after the addition of cystine, tryptophane, marmite, meat extract, blood, and urine (*see* Table I).

TABLE I.

A preliminary survey of nutrition.

Each test-tube contained 5 c.c. of salt and buffer mixture; 1 c.c. of hydrolysed gelatin (0·5 per cent); 0·2 c.c. of 0·1 per cent *l*-tryptophane or *l*-cystine; and 1 c.c. of extracts of Marmite or meat or urine or blood, each in a final concentration of 1×10^{-4}. Total volume 10 c.c. *See* text for the meaning of signs.

Serial number.	Medium.			GROWTH IN		
				24 hours.	48 hours.	120 hours.
1	Hydrolysed gelatin ..	..	..	0	0	tr.
2	,,	+ cystine	..	+	++	+++
3	,,	+ tryptophane ..	..	0	0	tr.
4	,,	+ cystine + Marmite	..	+	++	+++
5	,,	+ ,, + meat extract ..		+	++	+++
6	,,	+ ,, + mare's urine ..		+	++	+++
7	,,	+ ,, + rabbit blood ..		+	++	+++

The results recorded in Table I were obtained with all the strains tested, with one exception. Hydrolysed gelatin alone did not support growth, while full growth was obtained by adding cystine to it. Tryptophane could not replace cystine. Adding any of the natural sources did not stimulate the growth beyond that given by hydrolysed gelatin + cystine. The exceptional strain was I (virulent) which grew well in the absence of cystine; which fact was also confirmed in later experiments. These experiments indicate that the nutritional requirements of the plague bacillus are met entirely by the amino-acids of gelatin, with the single addition of cystine from outside. Further, they suggest that *accessory* growth factors are not involved in the nutrition of the organism.

II. *Hydrolysed gelatin as a suitable 'synthetic' medium.*—The sufficiency of hydrolysed gelatin + cystine for the growth of the bacillus, as shown in the above experiments, suggests that it can be used as a chemically defined or 'synthetic' medium for routine or research purposes. It is free from protein or forms of nitrogen precipitable with the usual protein precipitants. Growth tests were, therefore, made with all the strains on hydrolysed gelatin with or without cystine. The total N content of the medium was kept at 2·3 mg. per c.c. to conform with that of other laboratory media used for growing the organism. Without the addition of cystine there were found traces of growth with all the strains, but strain I (virulent) grew profusely as expected. The traces of growth noted are undoubtedly due to traces of mercapto compounds present in the gelatin used. All strains, however, grew luxuriantly when supplied with cystine. A few bacterial counts that were made showed that in comparison with acid-digest and peptone broths about the same

number of bacteria were produced by this medium in 48 hours from an equal size of inoculum. When incorporated with agar the medium produced much less growth, as estimated by the dry weight of the bacteria, than the other media in an equal period.

III. *Growth in an amino-acid mixture.*—The results obtained with hydrolysed gelatin are valid only if they can be repeatedly confirmed in more rigorous experiments with pure amino-acids. The amino-acids occurring in gelatin are: glycine, alanine, leucine, serine. phenylalanine, tyrosine, proline, hydroxyproline, aspartic acid, glutamic acid, histidine, arginine, and lysine (Dakin, 1920). Out of these, it was found that a mixture of seven amino-acids selected arbitrarily, with the further addition of cystine, supported fully the growth of all the strains used. The amino-acids are: (1) *dl*-alanine, (2) *dl*-leucine, (3) glycine, (4) *dl*-proline, (5) *dl*-phenylalanine, (6) *d*-lysine, (7) *d*-arginine, and (8) *dl*-cystine. The concentrations of the amino-acids are given in Table II :—

<div align="center">

TABLE II.

A pure amino-acid medium for the cultivation of the plague bacillus.

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The concentrations given refer to one isomer. Half the volume is made up of salt and buffer mixture.

Serial number.	Amino-acid.	Origin.	Molar concentration.
1	*dl*-alanine	Synthetic	$6{\cdot}7 \times 10^{-4}$ M.
2	*dl*-leucine	,,	$6{\cdot}7 \times 10^{-4}$ M.
3	glycine	,,	$6{\cdot}7 \times 10^{-4}$ M.
4	*dl*-proline	,,	$2{\cdot}5 \times 10^{-4}$ M.
5	*dl*-phenylalanine	,,	$2{\cdot}5 \times 10^{-4}$ M.
6	*d*-lysine 2HCl	Natural	$2{\cdot}5 \times 10^{-4}$ M.
7	*d*-arginine	,,	$2{\cdot}5 \times 10^{-4}$ M.
8	*dl*-cystine	Synthetic	$6{\cdot}7 \times 10^{-4}$ M.

Out of the eight amino-acids, arginine and lysine only are from natural sources, the rest being synthetic. However, from the next series of experiments it can be seen that both of these are non-essential and could be omitted from the mixture without effecting the growth. The ability of the plague bacillus to grow in this amino-acid mixture, therefore, confirms beyond doubt that accessory growth factors are not involved in the nutrition.

IV. *The essential amino-acids.*—The next series of experiments were directed towards finding out which amino-acid, when omitted *singly* from the complete mixture of eight amino-acids given in Table II, determined whether the medium supported the growth of the bacillus or not. Data on six of the strains used are given in Table III, the results of the other strains being similar. It was found that

TABLE III.

The determination of essential amino-acids.

	Strain I (virulent).				Strain I (avirulent).			
	24 hours.	48 hours.	72 hours.	96 hours.	24 hours.	48 hours.	72 hours.	96 hours.
With all 8 amino-acids ..	+	++	++	++	+	++	++	++
Lacking alanine ..	+	++	++	++	tr.	+	++	++
,, leucine ..	+	++	++	++	+	++	++	++
,, glycine ..	0	tr.	+	++	tr.	tr.	+	++
,, proline ..	0	0	0	0	0	0	0	0
,, phenylalanine ..	0	0	0	0	0	0	0	0
,, lysine ..	+	++	++	++	+	++	++	++
,, arginine ..	+	++	++	++	+	++	++	++
,, cystine ..	0	+	++	++	0	0	0	0

TABLE III—*contd.*

	STRAIN 120/5H (VIRULENT).				STRAIN 120/5H (AVIRULENT).			
	24 hours.	48 hours.	72 hours.	96 hours.	24 hours.	48 hours.	72 hours.	96 hours.
With all 8 amino-acids	+	++	++	++	+	++	++	++
Lacking alanine	+	++	++	++	+	++	++	++
,, leucine	+	++	++	++	+	++	++	++
,, glycine	0	tr.	+	++	0	tr.	tr.	++
,, proline	0	0	0	0	0	0	0	0
,, phenylalanine	0	0	0	0	0	0	0	0
,, lysine	+	++	+	++	+	++	++	++
,, arginine	+	++	++	++	+	++	++	++
,, cystine	0	0	0	0	0	0	0	0

Table III—*concld.*

	STRAIN 37 H (VIRULENT).				STRAIN 39 B (VIRULENT).			
	24 hours.	48 hours.	72 hours.	96 hours.	24 hours.	48 hours.	72 hours.	96 hours.
With all 8 amino-acids ..	+	++	++	++	+	++	++	++
Lacking alanine ..	+	++	++	++	+	++	++	++
,, leucine ..	+	++	++	++	+	++	++	++
,, glycine ..	tr.	+	++	++	tr.	+	++	++
,, proline ..	0	0	0	0	0	0	0	0
,, phenylalanine ..	0	0	0	0	0	0	0	0
,, lysine ..	+	++	++	++	+	++	++	++
,, arginine ..	+	++	++	++	+	++	++	++
,, cystine ..	0	0	0	0	0	0	0	0

Note.—See the text for the meaning and significance of the signs used in this and succeeding tables.

the requirements of all the strains, whether virulent or avirulent, are very uniform. The omission of proline or phenylalanine or cystine gives no growth whatever. These three amino-acids must be, therefore, considered indispensable for growth. Glycine is important since in its absence growth is delayed. The omission of any of the other amino-acids has no effect on growth, and these are, therefore, unessential. Cystine is not necessary for strain I (virulent) in confirmation with the previous observation.

V. *The interaction of amino-acids.*—In the above experiment the importance of each amino-acid has been evaluated by omitting it singly from the full amino-acid mixture. Such a procedure does not indicate how far the rôle of an amino-acid is dependent on the others, i.e., how far the amino-acids interact in the growth process. The determination of such interaction (or interdependence) is best done by omitting 2, 3, or more amino-acids in all possible combinations from the complete mixture. This was done according to the factorial design of experiment as given by Fisher (1935). For limiting the scope of the experiment it was assumed that interactions involving more than three amino-acids were unimportant experimentally. The results of an experiment with 120/5H (virulent) strain for the determination of 2-factor interaction are given in Table IV, and for 3-factor interaction in Table V :—

TABLE IV.

Determination of the interaction of two amino-acids.

	STRAIN 120/5H (VIRULENT).			
	24 hours.	48 hours.	72 hours.	96 hours.
1. With all 8 amino-acids	+	++	++	++
2. Lacking alanine + leucine ..	+	++	++	++
3. ,, alanine + glycine ..	tr.	+	++	++
4. ,, alanine + proline ..	0	0	0	0
5. ,, alanine + phenylalanine ..	0	0	0	0
6. ,, alanine + lysine ..	+	++	++	++
7. ,, alanine + arginine ..	+	++	++	++
8. ,, alanine + cystine ..	0	0	0	0
9. ,, leucine + glycine ..	tr.	+	++	++
10. ,, leucine + proline ..	0	0	0	0
11. ,, leucine + phenylalanine ..	0	0	0	0
12. ,, leucine + lysine ..	+	++	++	++

TABLE IV—*concld.*

	Strain 120/5H (virulent).			
	24 hours.	48 hours.	72 hours.	96 hours.
13. Lacking leucine + arginine ..	+	++	++	++
14. „ leucine + cystine ..	0	0	0	0
15. „ glycine + proline ..	0	0	0	0
16. „ glycine + phenylalanine ..	0	0	0	0
17. „ glycine + lysine ..	tr.	+	++	++
18. „ glycine + arginine ..	tr.	+	++	++
19. „ glycine + cystine ..	0	0	0	0
20. „ proline + phenylalanine ..	0	0	0	0
21. „ proline + lysine ..	0	0	0	0
22. „ proline + arginine ..	0	0	0	0
23. „ proline + cystine ..	0	0	0	0
24. „ phenylalanine + lysine ..	0	0	0	0
25. „ phenylalanine + arginine ..	0	0	0	0
26. „ phenylalanine + cystine ..	0	0	0	0
27. „ lysine + arginine ..	+	++	++	++
28. „ lysine + cystine ..	0	0	0	0
29. „ arginine + cystine ..	0	0	0	0

TABLE V.

Determination of the interaction of three amino-acids.

		STRAIN 120/5H (VIRULENT).			
		24 hours.	48 hours.	72 hours.	96 hours.
1.	With all 8 amino-acids	+	++	++	++
2.	Lacking alanine + leucine + glycine	tr.	+	++	++
3.	,, alanine + glycine + proline	0	0	0	0
4.	,, alanine + proline + phenylalanine	0	0	0	0
5.	,, alanine + phenylalanine + lysine	0	0	0	0
6.	,, alanine + lysine + arginine	+	++	++	++
7.	,, alanine + arginine + cystine	0	0	0	0
8.	,, leucine + glycine + proline	0	0	0	0
9.	,, leucine + proline + phenylalanine	0	0	0	0
10.	,, leucine + phenylalanine + lysine	0	0	0	0
11.	,, leucine + lysine + arginine	+	++	++	++
12.	,, leucine + arginine + cystine	0	0	0	0
13.	,, glycine + proline + phenylalanine	0	0	0	0
14.	,, glycine + phenylalanine + lysine	0	0	0	0
15.	,, glycine + lysine + arginine	tr.	+	++	++
16.	,, glycine + arginine + cystine	0	0	0	0
17.	,, proline + phenylalanine + lysine	0	0	0	0
18.	,, proline + lysine + arginine	0	0	0	0
19.	,, proline + arginine + cystine	0	0	0	0
20.	,, phenylalanine + lysine + arginine	0	0	0	0
21.	,, phenylalanine + arginine + cystine	0	0	0	0
22.	,, lysine + arginine + cystine	0	0	0	0

Taking the results from Tables III, IV, and V, the following conclusions can be drawn :—

    1. The omission of the three essential amino-acids, proline, phenylalanine, and cystine, in whatever combination, gives no growth. Each is independently capable of withholding growth, but all are together required to allow growth to take place.

2. Glycine is in a special category, since its omission irrespective of the presence or absence of other non-essential amino-acids always delays growth.

3. Alanine, leucine, lysine, and arginine are non-essential in any combination.

DISCUSSION.

The nutritional requirements of the plague bacillus are characterized by the complete absence of dependence on *accessory* growth factors. This is a remarkable fact in a purely parasitic bacterium that combines high virulence with extreme invasiveness. Most of the pathogenic forms investigated so far are dependent on some distinctive accessory factors : for example, nicotinic acid, aneurin, and uracil in *Staphylococcus* (Knight, 1937*a, b :* Richardson, *loc. cit.*) ; phosphopyridine nucleotides in *Hæmophilus influenzæ* (Lwoff and Lwoff, *loc. cit.*) and pimelic acid, nicotinic acid, and β-alanine for the diphtheria bacillus (Mueller, 1937*a, b ;* Mueller and Cohen, 1937).* Nutritional complexity is, however, still revealed by the fact that three different amino-acids are fully indispensable. Of these, the function of cystine is clearly to supply organic sulphur as the mercapto (—SH) group. The observation that one strain does not require it may indicate the possibility of adaptation towards simpler sulphur requirements. Proline, which is indispensable for all strains, has a pyrrole ring—
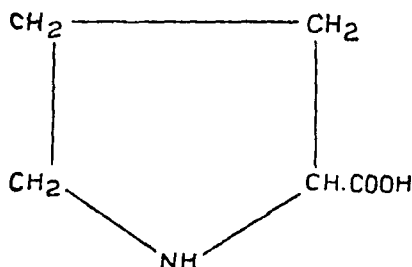
When it is considered that the blood pigments are constituted of pyrrole nuclei, the very profuse growth of the plague bacillus obtainable on blood agar may possibly have a nutritional basis. The third essential amino-acid is phenylalanine containing an aromatic ring—
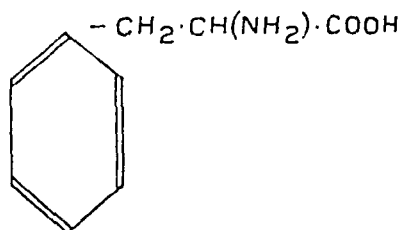
* Subsequent manometric experiments with washed suspensions of plague bacilli show that diphosphopyridine nucleotide (cozymase), flavin, etc., though unessential for growth, function as respiratory catalysts, in agreement with the current view that the rôle of several accessory factors is that of hydrogen-carriers in the oxidation systems of the bacterial cell.

According to the accepted theory, therefore, the synthetic powers of the plague bacillus do not extend to the mercapto (—SH) group, the pyrrole and the aromatic rings. Since these form essential structural units in proteins, they must be provided in the medium so as to enable the bacilli to assimilate them and grow. On the other hand the bacillus possesses the power of synthesizing the non-essential amino-acids, all of which have relatively simpler structures. Thus the two basic amino-acids, lysine and arginine, are fully dispensable as well as the two simple mono-amino-acids, alanine and leucine. Glycine, notwithstanding its very simple structure, has an important rôle in growth.

The metabolic reactions through which the indispensable molecular structures are assimilated *in toto* by the bacillus are of considerable theoretical interest, since there is little insight into such reactions at the present time. The interactions of amino-acids studied in this paper throw some light on the process. They show that the three essential amino-acids function interdependently. Further experiments on this aspect of metabolism are in progress.

There is little variation as regards nutrition between different strains and in particular between the virulent and the avirulent strains. A metabolic correlation with virulence, if any, in this bacillus, must be sought elsewhere than in amino-acid requirements.

The application of these facts to bacteriological practice can be briefly indicated. Two different chemically defined media are directly provided by the experiments: (1) hydrolysed gelatin + cystine and (2) proline + phenylalanine + cystine + glycine, which can be further enriched with non-essential amino-acids. The main facts of nutrition discovered, however, enable one to design media *ad infinitum*. In general, hydrolysates of simple proteins (such as gelatin or casein) in which the presence of essential amino-acids is ensured will prove to be the most useful. As a recent example of the special use to which such media can be put may be quoted the work of Pappenheimer (1937) who has isolated pure toxin produced by the diphtheria bacillus using a simple gelatin hydrolysate medium.

SUMMARY.

1. The nutritional requirements of several strains of plague bacilli have been studied with reference to amino-acids and accessory growth factors.

2. Three amino-acids, proline, phenylalanine, and cystine, are indispensable, and must be present in the medium before growth can proceed, while the presence of glycine, while not essential, is stimulatory.

3. Accessory growth factors (or bacterial vitamins) have no essential rôle in the nutrition of the plague bacillus.

4. The different virulent strains of the plague bacillus, obtained from different plague cases during epidemics, as well as the fully avirulent strains derivable from them by repeated culturing, have more or less identical nutritional requirements.

5. The preparation and utility of chemically defined protein-free media, such as hydrolysed gelatin + cystine, and of similar others that can be designed with the help of the above facts of nutrition, are emphasized.

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MOLLUSCUM CONTAGIOSUM AND ITS VIRUS.

BY

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THE infectious nature of *Molluscum contagiosum* was first demonstrated by Paterson in 1841 ; this was confirmed by Retzius in 1871, Vidal in 1878, Stelwagon in 1895, and others. In 1841, Henderson and Paterson described large oval hyaline structures in the superficial layers of epithelial nodules which were regarded as specific parasites and these well-defined structures have since been called molluscum bodies. Neisser considered these structures to be protozoa and similar to coccidia. In opposition to the parasitic view Hausemann, MacCallum, Unna, Benda, and others held that they represent a peculiar kind of cellular degeneration.

The later view of van Prowazek, Lipschutz and Hartmann is that they arise as the result of a specific reaction on the part of epithelial cells to the invasion of a virus.

In 1911 Lipschutz stated that the minute granular bodies, which appear in the intra-cellular vacuoles of the molluscum bodies, are the actual parasites of the disease and were named by him *Strongyloplasma hominis*.

More recently, Goodpasture and King (1927) suggested that the hyaline oval masses which constitute the molluscum bodies of Henderson are formed by a coalescence of the masses of intra-cellular granules and other intra-cytoplasmic material. This is brought about by desiccation, as the cells are forced outward. They are not formed, in his opinion, by an abnormal keratinization, as suggested by Lipschutz. They further consider that the minute bodies, the elementary bodies of Lipschutz, are not derived from extruded nucleoli, nor from any formed cytoplasmic constituent, but are morphologically consistent with a living, filter-passing virus.

The description of Goodpasture and King corresponds very closely with the account of the development of the virus inclusions in fowl-pox given by Ludford and Findlay in 1926. The virus was first shown to be filtrable by Juliusberg in 1905. He showed that the virus could pass through a Chamberland filter by successfully inoculating a human being with the filtrate. This was the first demonstration of a filtrable virus in connection with any human dermatosis. Findlay finds that it can pass through Berkefeld 5 and Chamberland L_1 filters. More recently, Wile and Kingery (1919) have shown filtrability of the virus through the finest Berkefeld filters.

Brain (1933) by means of the complement-fixation reaction and using suspensions of elementary corpuscles from curetted molluscum nodules, has demonstrated the presence of antibodies in the serum of a patient with multiple lesions.

At the present time, therefore, *Molluscum contagiosum* may be regarded as a typical example of a virus disease. The infective agent is readily filtrable, the disease exhibits a definite incubation period and the lesions in epithelial cells are accompanied by the formation of large cytoplasmic acidophile inclusion bodies.

Findlay said that the virus is pathogenic only for man. Attempts to infect laboratory animals, monkeys, and apes have all failed, neither fowls nor pigeons can be infected, nor is there any evidence to show that human *Molluscum contagiosum* is in any way related to the disease of birds known as avian molluscum, fowl-pox, or *Epithelioma contagiosum*.

It has been said that many viruses are prone to affect a particular type of cell. This fact is emphasized in the use of the expression ' tropism ' in reference to certain viruses. Some viruses are said to be neurotropic, namely herpes, rabies, poliomyelitis ; while others are epidermotropic, such as variola, vaccinia, fowl-pox, *Molluscum contagiosum*, infective warts, and pityriasis rosea.

Molluscum contagiosum is not very uncommon here and we decided to try if it were caused by a neurotropic virus and inoculated rabbits by cisterna puncture with a suspension of the diseased material in the same manner as is employed in rabies inoculation.

Technique of animal experiment.—Whenever possible we used material from newly-formed molluscum nodules. The nodule with the surrounding skin was cleansed with a mixture of equal parts of ether and alcohol and was snipped off with a pair of sterile curved scissors. It was then macerated in a sterile agate mortar into a paste, by means of a sterile agate pestle, with the addition of a little sterile normal saline. The paste was afterwards mixed with normal saline to form a suspension which was then filtered through a candle, Chamberland L_3, and the filtrate thus obtained was used as inoculum.

Inoculation.—Male rabbits were chosen as the most suitable animals and inoculation into the cisterna was performed. A sterile Collins-Roux syringe was used. First 0·2 c.c. to 0·3 c.c. of the inoculum was drawn into the syringe. With the animal under ether anæsthesia the region, where the syringe needle was to be inserted, was shaved and cleaned with ether-alcohol and the needle was introduced into the subdural space in the suboccipital region and 0·2 c.c. to 0·4 c.c. of clear spinal fluid was withdrawn, which indicates successful cisterna puncture. The inoculum and spinal fluid were then slowly injected into the cisterna. We found that 0·5 c.c. was the

maximum quantity that could be safely introduced because with a larger quantity there is danger of death. Only two out of 36 rabbits succumbed to intracranial hæmorrhage. In some cases unfiltered suspension was also taken as an inoculum and the results are given below.

RESULT OF ANIMAL EXPERIMENTS.

The total number of animal experiments, including controls, was thirty-six. In the first series twenty animals were utilized as follows: Six were inoculated with unfiltered molluscum nodule suspension and four with a filtrate of this suspension; three controls were inoculated with unfiltered normal human skin suspension, and three with its filtrate; and finally two rabbits were inoculated with unfiltered and two with filtered rabbit brain suspension. The results are shown in Table I :—

TABLE I.

Primary inoculations and controls.

Inoculum.	Number of rabbits.	Number remained healthy.	Number diseased.	Number recovered.	Number died.	Number positive by examination.
Unfiltered molluscum nodule suspension.	6	1	5	2	3 (2 killed and 1 died).	2
Filtered molluscum nodule suspension.	4	1	3	0	3	2
Unfiltered normal skin suspension.	3	2	0	0	1 of hæmorrhage.	0
Filtered normal skin suspension.	3	3	0	0	0	0
Unfiltered brain suspension.	2	2	0	0	0	0
Filtered brain suspension.	2	2	0	0	0	0

The first passage to another set of rabbits gave positive results. In this experiment the diseased rabbit was killed on the 10th day, the brain was removed aseptically, mixed with normal saline under strict aseptic conditions, and the inoculation of twelve rabbits was carried out with filtered and unfiltered suspension of

the brain. Similarly, a second passage was performed by taking the brains of this second group of rabbits and inoculating four other rabbits, as shown in Table II :—

TABLE II.

Results of sub-inoculation.

	Inoculum.	Number of rabbits.	Number remained healthy.	Number diseased.	Number recovered.	Number died.	Number positive by examination.
1st passage	Unfiltered ..	6	1	5	2	3 (one of hæmorrhage).	2
	Filtered ..	6	0	6	2	4	3
2nd passage	Unfiltered ..	2	0	2	0	2	2
	Filtered ..	2	0	2	0	2	2

SYMPTOMS IN EXPERIMENTAL ANIMALS.

It appears from the observation of 13 positive animals which were inoculated with molluscum nodules and with infected brain, the majority in the first passage become sick within 2 to 3 days. After 48 hours the animal looks unwell, bending its head to the left side with full stretching of the right hind leg and the eyes have an anxious look. It does not take food readily. It was thought that the bending of the head and sickness might be due to the local injury caused by the puncture of the needle during inoculation but no such inflammatory process could be found subsequently. Gradually the movements became limited and the animal could not hop and jump as it normally does, but it pulled itself along by advancing one hind leg ahead of the other. The front legs are not affected at first, but on the 6th day these also became paraplegic. There was a spastic paraplegia of the hind legs, which were contracted, rigid, and resistant to passive movements. On the 8th day there was a flaccid type of paraplegia of the hind legs and spastic paraplegia of the front legs, which dragged the whole body forward; finally, this stopped and the animal could not move. Unfortunately we could not observe the animals at the time of death as all except one died at night, but we noticed respiratory difficulty with shallow breathing, the previous evening. It may be possible that

the respiratory centre is also paralysed at the time of death. The animal gets spasm off and on, especially when disturbed during the illness. Some animals, in which the front legs did not become affected, recovered within 2 to 3 weeks. One of those 'recovered' rabbits developed gradual cachexia which became very pronounced at the 3rd week and the animal died. The peculiarity about the first and second passages is that the animals developed the above symptoms within 24 to 48 hours and died on the 4th to 6th day, prominent symptoms being severe spasms of the extremities and respiratory failure at the time of death.

Post-mortem appearances :—

1. Skin—normal.
2. Spleen, liver, lungs, heart, and kidneys are highly congested.
3. Brain—there is marked congestion of the brain, the blood vessels being very prominent and distended with blood. The membrane is congested and swollen. On section the brain is œdematous and minute hæmorrhages are seen here and there. Very little exudation is present. The cord is also congested.

Histopathology of brain.

In the substance of the brain the changes, which are confined mainly to the pyramidal cells, consist of various stages of degeneration, the cells are pyknotic, their nuclei cannot be made out clearly, the Nissl granules have disappeared and definite signs of neurophagy are present. In places, the cells appear as black masses, scattered amongst their comparatively less-injured neighbours. The degenerative process has picked out cells indiscriminately and a damaged cell may be seen alongside a healthy one. There is no neuroglial proliferation. No change can be made out in the white matter. The pial membrane is congested, its blood vessels are engorged with blood and there is some round-celled infiltration. These features are limited strictly to the meninges. There are minute hæmorrhages here and there in the brain substance.

Complement-fixation reaction.

The technique of complement-fixation was employed according to the advice given by Major S. D. S. Greval, I.M.S., who helped us greatly in selecting the proper antigen and also in obtaining a suitable titration.

Antigen.—First the saline suspension of molluscum nodules was tried but it was frequently anticomplementary and when diluted sufficiently to overcome this defect it was poor in fixing power. Then an alcoholic extract of the same material was taken as an antigen but it gave conflicting results and after several modifications including the addition of cholesterol it was found still unreliable and its use was discontinued.

Next we thought of preparing antigen from the infected rabbit brains which has been kept about six months in suspension in half per cent carbolized normal saline in an ice-chest. According to the suggestion of Major Greval the suspension was filtered and the filtrate was taken as antigen; this proved to have no fixing

power, though it was not anticomplementary and not hæmolytic. Then the suspension was filtered through a filter-paper and it was found to be of the same nature.

Next we took the brain substance on the filter-paper and prepared an alcoholic extract from it. The extract was used as an antigen and it was neither anticomplementary nor hæmolytic. It also showed good fixing power when treated with serum of *Molluscum contagiosum* cases.

The anticomplementary activity of the antigens was invariably tested before use by titrating them in the presence of 1 M. H. D. of complement. An antigen was considered satisfactory if it did not inhibit the action of 1 M. H. D. of complement when present in twice the concentration used in the test.

Sera.—The sera investigated were obtained from cases of *Molluscum contagiosum* in various stages the majority being of long duration and a few convalescent ones. Control sera were obtained from individuals suffering from infective warts, herpes zoster, lupus vulgaris, tinea cruris, and syphilis of which one each of herpes zoster, lupus vulgaris, and tinea cruris gave positive results; there was a history of 'warty growth' about two years back in the tinea cruris case.

Quantities and time used in the test

0·25 c.c. 1 in 5 diluted serum is mixed with 0·25 c.c. titrated antigen and 0·25 c.c. complement-dilution representing 2 M. H D. This mixture is kept at room temperature for half an hour, and in an incubator at 37°C. for half an hour. After the above period of fixation 0·25 c.c. of 3 per cent sensitized sheep-cell suspension is added and the whole is incubated at 37°C. for thirty minutes. We intend to modify the period of fixation in our further work according to the indication in the work of Bedson and Bland (1929) that the union between virus and antibody takes place slowly, and this suggested that better complement-fixation might be obtained if the period of fixation were prolonged. This naturally cannot be done at 37°C. because complement deteriorates fairly rapidly at this temperature.

TABLE III.

The results of complement-fixation reaction.

Disease.			Total number of cases.	Positive.	Doubtful positive.	Negative.
Molluscum contagiosum		..	24	20	3	1
Infective warts	..	..	8	0	2	6
Herpes zoster	..	..	6	1	1	4
Lupus vulgaris	..	..	5	1	0	4
Tinea cruris	..	..	5	1	0	4
Syphilis ..	..	..	5	0	1	4

PLATE I.
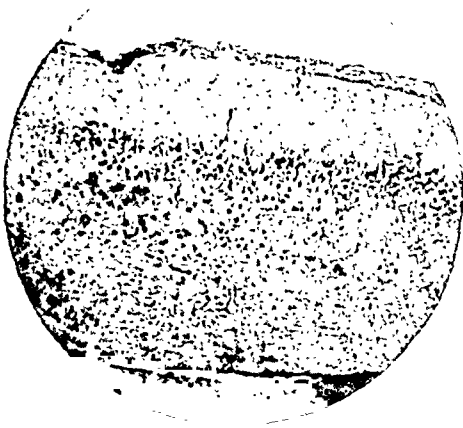
Fig. 1. Section of parietal lobe of the brain ($\times$ 80 approx.) showing thickened pial membrane and degenerated pyramidal cells.
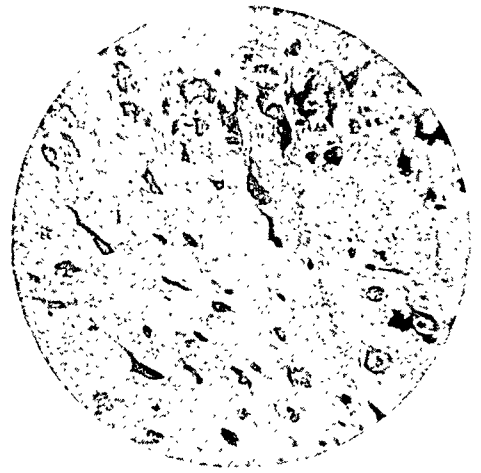
Fig. 2. Section of same ($\times$ 450 approx.) showing pyknotic and degenerated pyramidal cells.
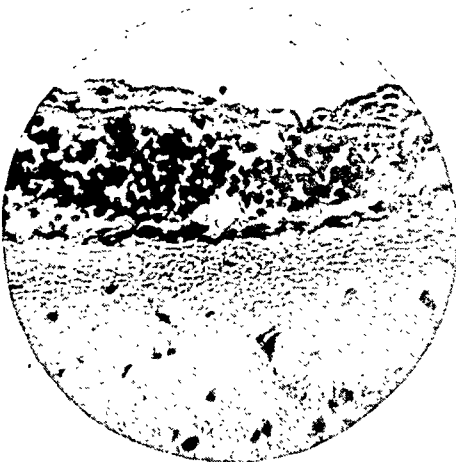
Fig. 3. Section of same ($\times$ 450 approx.) showing the thickened pial membrane with its blood vessels engorged with blood and also hæmorrhages.
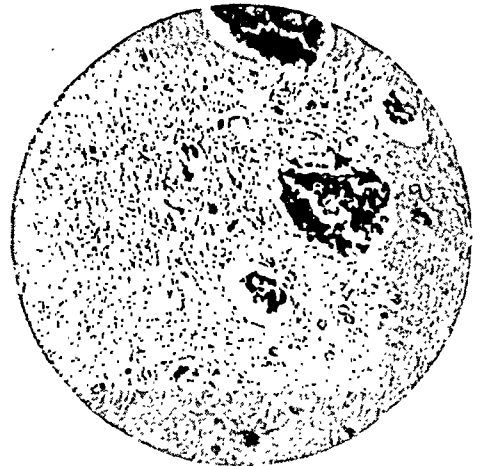
Fig 4. Section of the same ($\times$ 450 approx.) showing engorged blood vessels in the white matter with extravasation of blood.
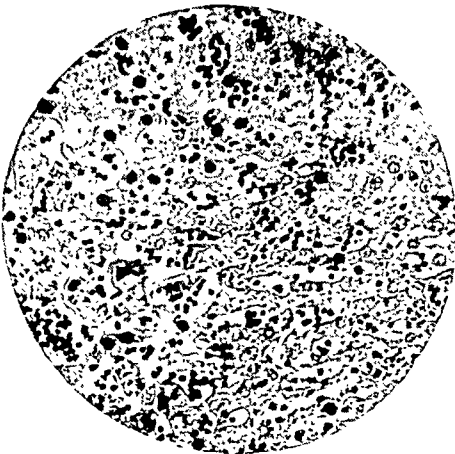

Fig. 5. Section of liver ($\times$ 450 approx.) showing marked congestion.
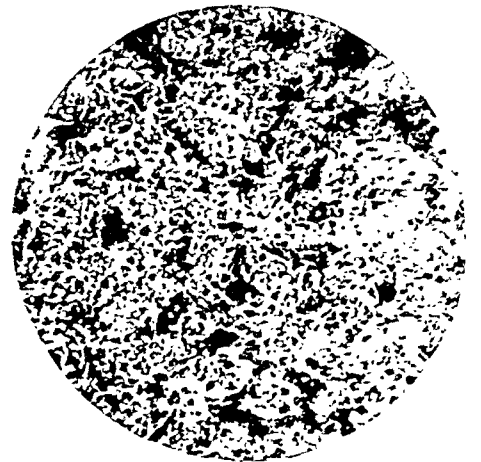
Fig. 6. Section of spleen ($\times$ 450 approx.) showing congestion and hæmorrhages.

Thus, by the serologic test we are able to demonstrate that the disease manifested by the rabbits was caused by the same virus as that of *Molluscum contagiosum* in man. Positive complement-fixation reactions between the blood serum and the alcoholic extract of the brain of the infected animal were obtained in most cases.

The three control sera which gave positive-fixation came from herpes zoster, lupus vulgaris and tinea cruris and one of them may have had an attack of *Molluscum contagiosum* some time previously.

SUMMARY.

1. Rabbits inoculated with the filtrates obtained from the suspension of molluscum nodules by cisterna puncture exhibited a characteristic clinical picture, showing the transmission of infection from human beings to animals.

2. Brains of positive rabbits show marked pathological changes, which seem to be peculiar to the molluscum virus.

3. Its passage through animals was repeatedly successful. The successful transfers made with the filtrates of brain tissue suspension point to an invisible filtrable virus as the cause of the disease.

4. Serologic tests with the antigen prepared from the infected brain are of diagnostic importance.

Goodpasture (1929) said that there was an interesting variation in the cellular affinity of the three viruses, namely vaccinia, fowl-pox, and *Molluscum contagiosum*, brought out in the case of two of them by infection on the chorio-allantoic membrane of the chick embryo. *Molluscum contagiosum* had not yet been successfully engrafted upon a host other than man, but so far as one knows it affects only and especially ectodermal epithelial cells in their natural host and it would probably require a culture of ectodermal epithelium to initiate its growth.

We have had partial success in propagating the molluscum virus on the chorio-allantoic membrane of a developing chick embryo by the inoculation of the filtrate of a saline suspension of the molluscum nodules following the method of inoculation described by Burnet (1936). The details of this work will be published later.

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STUDIES ON BASAL METABOLISM IN BOMBAY.

Part I.

BY

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INTRODUCTION.

THE problem of basal metabolism has in recent years aroused a certain amount of interest among the nutrition workers in India. An extensive study of the subject has become necessary in view of the fact that the basal metabolic rate (B.M.R.) is an important factor in the determination of caloric requirements. The standards of basal metabolism as established in America and in Europe do not apply to people living in the tropics. In India the dietetic, climatic, occupational, and other factors which are known to influence the basal metabolism are quite different from those prevailing abroad and they are so much at variance all over this country, that an extensive series of regional investigations are necessary for calculating the B.M. standards for people resident in the different parts of India.

Mukherjee (1926) collected data from his observations on 15 Bengalee male medical students between the ages of 22 and 27 years at Calcutta, and found the basal metabolism to be 9 per cent below the Aub and DuBois standards. Sokhey (1927) using a Collin's chain compensated gasometer with Haldane's air analysis apparatus measured the B.M.R. of 21 male medical students in Bombay and reported a variation of 10 to 23 per cent below the Aub and DuBois standards in 15 of his subjects. In Madras, Mason and Benedict (1931) working on 53 women students and teachers found a deviation of 16·9 and 17·2 per cent below Harris-Benedict and Aub

and DuBois standards respectively. Mukherjee and Gupta (1931) investigated the B.M.R. of 18 Bengalee medical students at Calcutta and observed a deviation of 13·3 per cent below the Aub and DuBois standards. Banerji (1931) found the basal metabolism of 145 prisoners of Lucknow districts jail to be 7 per cent below Aub and DuBois standards. Bose and De (1934) carried out observations on 30 men and 30 women at Calcutta and their results were within 5 per cent of the standards of Aub and DuBois. Krishnan and Vareed (1932) also reported values for men and women considerably lower than the Harris-Benedict and Aub-DuBois standards. Rahman (1936) conducted experiments on 32 young men at Hyderabad and found a deviation of 8·7 per cent below the Aub-DuBois standards.

The present communication is a preliminary report of the observations of B.M.R. recorded on 76 healthy adults, 24 men and 52 women, residents of Bombay. In order to lay down standards for basal metabolism and to assess the relative import-ance of the different factors influencing metabolism a large number of observations are absolutely indispensable. The work is being continued and the data obtained from all future experiments will serve as a supplement to the present series of results for subsequent statistical treatment.

EXPERIMENTAL.

In the present publication the observations on 76 subjects are reported. Each subject was experimented on on three separate days and on each day three records, each of 10 minutes period, were taken at intervals of 15 minutes.

Subjects selected.—The subjects selected were healthy medical students and members of the staff of the Medical College and nursing staff of the K. E. M. Hospital, Bombay. They belonged to different communities of both sexes and were of a homogeneous group as far as age and living conditions were concerned, some being vegetarians and others non-vegetarians. They were all examined a few days before the tests to eliminate those that revealed some abnormal clinical conditions. A few days before the commencement of the tests, the subjects were shown the apparatus and the whole test demonstrated to them in order to minimize any mental disturbance resulting from the novelty of the apparatus used.

The men and women subjects selected were between the ages of 18 and 35 years. These subjects being of a literate class gave correct information of their ages and hence results obtained could safely be compared with the standards laid down for the corresponding age groups. Further information concerning the physical measurements is given in Table I.

Apparatus and technique.—The apparatus used was Sanborn's graphic meta-bolism tester. In determining the basal metabolism the subject lies on a couch with his nose clamped, and breathes in and out of the instrument through a mouth-piece and two wide bore tubes (inspiratory and expiratory), provided with valves. The main part of the instrument consists of a bell-type spirometer. At the commence-ment of the experiment sufficient oxygen is admitted from a cylinder to raise a pointer on the spirometer bell to a known mark upon an adjoined scale calibrated to oxygen volumes, the accuracy of which has been checked in the laboratory. Due precautions are taken to test for leakage and for the presence of carbon dioxide in the spirometer bell. As oxygen is consumed the spirometer falls and from the heights of the recorded graph at the beginning and end of the experiment the oxygen

consumption per minute is obtained. The volume of oxygen used includes correction for water vapour and for temperature of 21°C. For barometric pressure and other temperatures corrections are made by the use of tables supplied with the apparatus. The heat production is found by reference to the tables on the assumption that the average R.Q. in the post-absorptive stage is 0·82.

The data for the physiological and physical measurements are given in Table I:—

TABLE I.

Data.	24 men.	52 women.
A. Age in years and months.		
Maximum	35/6	29/10
Minimum	18/7	18/5
Average	22/8	22/-
B. Weight (without clothes) in kg.		
Maximum	71·3	62·2
Minimum	42·7	30·4
Average	52·5	44·7
C. Standing height in cm.		
Maximum	183·6	164·3
Minimum	152·2	140·5
Average	166·0	152·7
D. Sitting height in cm.		
Maximum	95·3	86·1
Minimum	80·5	74·7
Average	86·2	79·6
E. Pirquet Index or Pelidisi.		
Maximum	105·5	105·8
Minimum	85·2	86·2
Average	93·4	92·3

TABLE I—*contd.*

Data.	24 men.	52 women.
F. Respiration rate.		
Per minute during the experiment	17	17
G. Pulse rate per minute.		
Average before the test	65	79
Average during the test	65	70
H. Blood pressure.		
Average systolic	104	102
Average diastolic	63	64
I. Vital capacity in c.c.		
Maximum	4,600	2,900
Minimum	1,500	900
Average	2.552	1,622
J. Barometric pressure in mm. Hg.		
Maximum	766	765
Minimum	754	755
Average	760	761
K. Room temperature in °F.		
Maximum }	91	91
Minimum } dry bulb	75	74
Average }	83·6	83·5
Maximum }	86	87
Minimum } wet bulb	69	58
Average }	79	74·8

TABLE I—*concld.*

Data.	24 men.	52 women.
L.　Percentage of humidity.		
Maximum　..	100	92
Minimum　..	50	31
Average　..	76	69

During the first few weeks of the investigation, the subjects came from home to the laboratory in the morning at 7 a.m. having taken their last meal 12 to 13 hours before the tests. Instructions were given to the subjects to eat a light dinner, not high in protein, and retire early to get a good night's rest. On the morning of the test, no bathing or breakfast, tea, milk or coffee was allowed and exertion of all types avoided, the subjects starting in good time from home so as to reach the laboratory without hurrying. The tests were conducted 45 minutes after the subjects had completely rested in the experimental room. The pulse and respiration were frequently recorded during the test. Blood pressure was also recorded before the beginning of the test. In the later tests the subjects slept overnight in the experimental room, and it was found that the B.M.R. was slightly lower as compared to that obtained when they came from home. The data published in this paper are from records of subjects who slept overnight in the experimental room. Every possible effort was made to keep the subjects in perfect mental rest and muscular repose, and any excitement could be detected from the pulse, the respiration rate, and the type of spirographic tracings. The subjects were tested on three different days, three observations being taken each day. From each of the three-day series the average of the two lowest figures that agreed within 5 per cent were used for the estimation of B.M.R. for that series.

The average oxygen consumption on the second and third days was found to be lower than that observed on the first day, both in case of men and women (Tables II and III). This is to be attributed to greater repose and tranquillity ensured in the later series of experiments rather than to fatigue which was obviated by leaving an interval of at least three days between the consecutive series of observations recorded on three different days. In calculating the standard B.M.R. the mean of the second and third day series was taken into account and the results of the first day series were discarded for this purpose. In Tables II and III are given the results of the third series of tests on men and women respectively. The averages of the first and second series are given at the bottom of the tables for purposes of comparison.

TABLE II.

Basal metabolism of normal men in Bombay, series III.

Record number.	Age.	Body-weight, kg. (without clothes).	Height, standing, cm.	O₂ consumption per minute, c.c.	Heat production in calories.			Percentage deviation from standards.		
					For 24 hours.		For 1 hour.	Mayo Clinic.	Harris and Benedict.	Aub and DuBois.
					Total.	Per kg.	Per sq. m.			
4	19	53·8	171·2	187	1,299	24·1	33·4	—21·1	—15·2	—18·5
18	19/11	57·9	174·1	213	1,480	25·6	36·5	—13·5	—7·8	—7·6
12	19/11	45·4	172·2	184	1,278	28·1	35·5	—14·4	—8·3	—13·4
14	20/6	49·0	168·2	202	1,403	28·6	38·0	—7·3	—3·1	—3·8
23	20/6	39·5	159·9	165	1,146	29·4	35·4	—14·5	—10·1	—10·4
10	20/7	43·4	158·4	153	1,063	24·4	31·0	—23·9	—19·7	—21·5
13	20/7	51·8	159·3	195	1,355	25·2	37·2	—11·8	—6·1	—5·8
1	20/10	61·8	163·7	195	1,355	21·9	34·0	—17·7	—15·7	—13·9
9	20/10	71·3	168·9	220	1,529	21·5	35·6	—15·4	—14·2	—9·9
11	20/10	54·5	174·4	175	1,216	28·1	30·7	—25·2	—22·2	—22·3
21	21	57·7	165·4	204	1,417	24·6	36·5	—10·9	—8·8	—7·6
3	21/1	46·3	163·0	194	1,348	29·1	38·2	—7·6	—1·9	—2·3
20	21/2	71·3	183·6	223	1,549	21·7	33·8	—18·9	—14·9	—14·4
8	21/7	55·9	173·6	219	1,522	27·2	38·4	—5·6	—3·3	—2·8

24	22/3	44·0	159·4	171	1,188	27·0	34·6	—15·8	—11·0	—12·4
7	22/5	43·1	159·0	177	1,230	28·5	36·6	—10·6	—6·1	—7·3
15	22/9	66·8	158·7	202	1,403	21·0	34·8	—14·8	—13·2	—11·9
19	23	54·3	174·2	185	1,285	23·7	32·7	—21·6	—15·2	—17·2
25	24	46·6	152·2	164	1,139	24·4	34·2	—15·9	—12·4	—13·4
2	24/4	62·7	169·9	200	1,390	22·2	33·9	—15·6	—14·2	—14·2
17	25/8	46·8	163·7	187	1,299	27·8	37·1	—9·7	—5·1	—6·1
6	26/5	42·2	158·1	167	1,160	21·8	35·0	—15·6	—8·8	—11·4
22	35/6	60·4	169·9	186	1,292	21·4	31·8	—17·3	—14·6	—19·5
5	35/6	47·2	165·4	141	979	20·7	27·2	—28·7	—24·6	—31·1
Averages.										
Series III.	22/11	53·0	166·1	188	1,305	24·9	34·7	—15·5	—11·5	—12·5
,, II.	22/8	52·5	166·1	186	1,288	24·6	34·3	—16·4	—11·9	—13·3
,, I.	22/5	52·2	165·9	193	1,342	25·6	35·8	—13·0	—8·3	—9·4

TABLE III.

Basal metabolism of normal women in Bombay, series III.

Record number.	Age.	Body-weight, kg. (without clothes).	Height, standing, cm.	O₂ consumption per minute, c.c.	Heat production in calories.			Percentage deviation from standards.		
					For 24 hours.		For 1 hour.	Mayo Clinic.	Harris and Benedict.	Aub and DuBois.
					Total.	Per kg.	Per sq. m.			
7	18/10	40·2	150·5	135	938	24·3	29·8	−17·1	−22·1	−19·4
4	19/3	43·4	157·1	152	1,056	24·3	31·6	−13·6	−16·9	−16·8
12	19/5	42·5	153·6	155	1,077	25·3	33·3	−7·7	−13·7	−12·4
23	19/7	46·8	154·5	153	1,063	22·7	31·0	−14·0	−18·0	−18·4
9	19/10	52·2	147·9	156	1,084	20·8	31·2	−14·3	−18·1	−17·9
20	20/3	40·9	143·7	138	959	23·4	31·4	−14·3	−21·6	−15·4
10	20/4	40·0	155·9	133	924	23·1	28·7	−19·8	−24·7	−22·4
11	20/5	46·8	162·1	146	1,014	21·7	28·7	−19·7	−21·1	−22·4
15	20/7	37·7	156·2	157	1,091	28·9	35·0	−2·5	−9·2	−5·4
3	20/7	52·5	151·3	164	1,139	21·7	32·1	−9·4	−14·1	−13·2
17	20/9	42·9	148·1	159	1,105	25·7	34·6	−4·2	−11·5	−8·9
25	21/3	37·2	152·4	146	1,014	27·3	33·3	−8·2	−14·7	−10·0
8	21/4	42·7	156·7	154	1,070	25·1	32·3	−10·5	−14·5	−12·7
19	21/5	39·5	154·2	155	1,077	27·3	33·7	−6·6	−11·1	−11·3
18	21/9	31·8	144·4	127	882	27·7	31·4	−10·6	−20·3	−15·1

−10·8	−13·3	−10·4	33·0	27·3	1,077	155	159·0	39·5	22	13
−10·8	−11·9	−8·1	33·0	23·0	1,174	169	154·7	51·3	22/2	21
−15·1	−20·1	−12·5	31·4	23·8	972	140	145·6	40·9	22/2	22
−5·9	−9·7	−3·0	34·8	26·7	1,112	160	151·8	41·6	22/4	2
−7·8	−13·0	−8·4	34·1	26·0	1,063	153	149·0	40·9	22/7	26
−7·5	−8·8	−5·0	34·2	25·6	1,174	169	155·8	45·9	22/11	5
−17·0	−17·5	−14·7	30·7	20·4	1,084	156	152·1	53·1	23/2	24
−12·4	−14·6	−10·6	32·4	23·0	1,112	160	153·6	48·4	23/7	27
−5·1	−6·8	−1·8	35·1	27·2	1,112	160	150·6	40·9	24/1	14
−7·4	−3·6	−3·1	35·2	23·2	1,292	186	155·6	55·6	26/2	6
−10·5	−10·6	−7·5	33·1	23·6	1,112	160	151·3	47·2	29/10	16
−20·3	−17·4	−17·7	30·3	22·4	1,098	158	164·3	49·0	18/6	53
−16·3	−17·9	−15·6	31·8	24·5	1,014	146	152·6	41·3	19/2	47
−17·9	−17·0	−15·0	31·2	24·2	1,056	152	160·9	43·6	19/2	55
−15·5	−17·3	−12·1	32·1	25·0	1,007	145	149·6	40·2	19/3	43
−16·6	−19·1	−13·3	31·7	25·7	952	137	148·9	37·0	19/11	42
−14·5	−11·4	−9·9	32·5	24·5	1,139	164	161·4	46·5	20	31
−12·4	−13·7	−11·2	32·4	24·8	1,105	159	158·4	44·5	20	36
−17·0	−21·0	−14·8	30·7	23·1	958	138	147·6	41·5	20	60
−21·1	−25·4	−19·0	29·2	22·3	917	132	150·5	41·1	20/2	41
−16·5	−20·9	−14·5	30·9	23·4	979	141	149·8	41·8	20/11	56
−21·9	−23·0	−19·6	28·9	20·2	1,028	148	155·2	50·9	21/7	54
−6·7	−11·6	−4·7	34·5	25·1	1,119	161	150·5	44·5	21/11	52

Table III—*concld.*

Record number.	Age.	Body-weight, kg. (without clothes).	Height, standing, cm.	O₂ consumption per minute, c.c.	Heat production in calories.			Percentage deviation from standards.		
					For 24 hours.		*For 1 hour.*	Mayo Clinic.	Harris and Benedict.	Aub and DuBois.
					Total.	Per kg.	Per sq. m.			
39	22	62·2	152·3	162	1,126	18·1	29·7	—16·9	—21·6	—19·7
50	22/3	40·9	145·5	136	945	23·1	30·8	—15·0	—22·4	—16·8
64	22/4	46·8	144·1	147	1,021	21·8	31·5	—12·5	—19·8	—14·9
63	22/6	30·6	145·0	128	889	29·1	32·4	—9·8	—20·3	—12·4
48	22/10	46·5	149·7	150	1,042	22·4	31·4	—13·8	—17·0	—15·1
57	*23/6*	*59·7*	*162·7*	*156*	*1,084*	*18·2*	*27·9*	*—24·3*	*—23·0*	*—24·6*
59	24/1	55·0	162·4	187	1,299	23·6	34·4	—3·6	—4·6	—7·0
29	24/3	35·6	152·8	130	903	25·4	29·8	—16·7	—22·3	—19·4
44	24/5	39·0	154·2	145	1,007	25·8	32·1	—11·6	—15·2	—13·2
30	24/7	46·3	157·1	147	1,021	22·0	29·7	—17·4	—19·5	—19·3
33	25	47·2	159·5	166	1,153	24·4	32·9	—7·8	—9·9	—11·1
45	25/4	43·8	157·2	155	1,077	24·6	32·1	—9·9	—13·1	—13·2
37	26/3	52·5	145·7	155	1,077	20·5	31·6	—11·9	—17·5	—14·6
40	28/1	40·0	140·5	156	1,084	27·1	36·4	+2·6	—6·3	—1·6
Averages.										
Series III.	22	44·5	152·8	152	1,055	24·0	32·0	—11·2	—15·9	—14·1
,, II.	22	45·3	152·8	152	1,060	24·1	32·1	—10·9	—15·8	—13·5
,, I.	21/11	44·3	152·6	156	1,087	24·8	33·0	—8·5	—13·4	—11·4

DISCUSSION.

The average oxygen consumption was found to be 193, 186, and 188 c.c. for men and 156, 152, and 152 c.c. for women in the 1st, 2nd, and 3rd series respectively. The oxygen consumption in the first series of observations was higher than that observed in the second and third series. The observations for the two latter were, however, in good agreement. The values for basal metabolism obtained in these experiments have been compared with the Mayo Clinic, Harris-Benedict, and the Aub-DuBois standards. The average of each series has been found to be low when compared with any of these standards. The Mayo Clinic standards have been obtained by finding the averages of a number of readings taken on one day only (*see* DuBois, *loc. cit.*) and hence are likely to give somewhat high prediction values. The Aub-DuBois standards are, according to Krogh (1925), higher by about 6 per cent. Benedict (1928) also believes that the basal metabolism standards for women should be lower by about 5 per cent than the Harris-Benedict standards in use ; he further suggests that the Aub-DuBois standards for women should be lower by 12 per cent than the corresponding standards for men. Thus, it will be apparent that on account of the prevailing uncertainty about the current standards it becomes difficult to draw any definite conclusions based on the observed deviations. A comparison of the Indian data on the oxygen consumption and the heat production will perhaps be more useful. The values for these reported here are found to be in good agreement with those reported by some of the other workers in India. Table IV shows the comparative Indian values referred to above.

The figures found in the table show good agreement between the various results reported from different places. These data present a possibility of obtaining a uniform standard of basal metabolism for Indians in general. Banerji (*loc. cit.*) and Rahman (*loc. cit.*) who have obtained higher values are inclined to think that these might be due to the influence of the different climatic conditions in maritime places such as Calcutta, Bombay, and Madras on the one hand and inland places such as Lucknow and Hyderabad on the other ; the climate inland is drier than on the coast. Banerji has not given figures for the actual oxygen consumption or heat production, but the figures obtained by Rahman can be compared with those of Krishnan and Vareed (*loc. cit.*). When these authors calculated the average heat production by taking all the readings, they obtained a figure (36·7 calories per sq. m. per hour) higher than that (34·8 calories per sq. m. per hour) calculated by using only the minimum readings observed with each subject. Since the averages calculated by Rahman have been obtained by using all the readings from each subject, and since these values agree with the higher values of Krishnan and Vareed (*see* the table), it seems probable that the average basal metabolism of men in Hyderabad could be actually lower than that reported by him. It is also possible that the high values obtained by Banerji (*loc. cit.*) and Bose and De (*loc. cit.*) might be due to similar reasons. In case of women the values for basal metabolism reported by us, although slightly higher, agree fairly well with those reported by Mason and Benedict (*loc. cit.*) and by Krishnan and Vareed.

So far as the present investigation is concerned the oxygen consumption on the first day of the experiment was, as has been stated elsewhere, found to be somewhat higher than on the second and the third days. The agreement between the observations on the two latter days was good and hence it is felt that the values

TABLE IV.

Authors.	Place.	MEN.					WOMEN.				
		Number of subjects.	Oxygen consumption per minute, c.c.	Heat production per sq. m. hour, calories.	Percentage deviation: Harris and Benedict.	Percentage deviation: Aub and DuBois.	Number of subjects.	Oxygen consumption per minute, c.c.	Heat production per sq. m. hour, calories.	Percentage deviation: Harris and Benedict.	Percentage deviation: Aub and DuBois.
Mukherjee and Gupta (1931) ..	Calcutta	18	186	34·26	..	−13·3	..	..	..	..	..
Mason and Benedict (1931) ..	Madras	..	..	..	..	..	54	150	31·2	−16·9	−17·2
Krishnan and Vareed (1932) ..	Madras	58	..	34·8	−10·8	−12	15	..	31·0	−18·2	−16·2
			..	36·7	−5·8	−7·2		..	32·3	−15·5	−13·2
Banerji (1931) ..	Lucknow	145	..	..	..	−6·9	..	..	..	..	..
Bose and De (1934) ..	Calcutta	30	209	..	..	±5	30	181	..	..	±5
Rahman (1936) ..	Hyderabad (Deccan).	32	206	36·7	−6·8	−8·7	..	..	..	..	..
Rajagopal (1938) ..	(Coonoor)	26	192	..	−8·9	−12·5	..	..	..	..	..
Niyogi, Patwardhan and Mordecai (present communication).	Bombay	24	187	34·5	−11·7	−12·9	52	152	32·05	−15·9	−13·8

obtained by finding the mean of the 2nd and 3rd day observations will give very nearly a true figure for the basal metabolism of men and women in Bombay.

Numerous factors are known to influence the basal metabolism. In addition to age, sex, height, and weight, several characteristics of a locality may affect the metabolism :—

(a) The climate may play an important rôle. In all basal metabolism experiments and in all summations of basal metabolism data the possible effect of seasonal variation cannot entirely be disregarded. The conclusions of the different workers on the seasonal variability in metabolism are at variance. Ahmad, Lal and Roy (1938) obtained apparently contradictory results in a series of experiments carried out in air-conditioned rooms where the temperature and relative humidity were low. They found a decrease in the rate of heat production when the subjects entered the air-conditioned room from the warm and humid environment. In studying this problem one has several factors to consider, viz., the temperature, humidity, and the influence of sunlight. Indeed the various factors making up a tropical climate should be carefully considered in such analysis. In order to get a complete picture of the effect of season on metabolism it is intended to make observations on each individual on three different days each month throughout an entire year.

(b) The diet with particular reference to the protein intake is another important factor, for with a low protein intake, a low metabolism might be expected (Williams and Benedict, 1928). According to Best and Taylor (1937) the B.M.R. of vegetarians shows no significant difference from that of meat eaters ; the nature of the diet would seem to have little influence on metabolism. In view of these contradictory statements it would be worth while to carry out further work on the metabolism of the vegetarians and non-vegetarians.

(c) The importance of taking blood pressure into account in metabolism studies cannot be overestimated. Thus, as a result of under-feeding, a group of 12 men were found to have a low B.P. and a low metabolism (Benedict, Roth and Smith, 1919). The B.P. curve of a man who fasted 31 days ran parallel to the curve for heat production (Benedict, 1915). The B.P. of the Chinese in general have been found to be low and the nutrition observations on Chinese women have shown a consistently low metabolism. For further work, particularly in racial metabolism, observations on the prevailing blood pressure are necessary with a view to establish its correlation, if any, with metabolism.

(d) In addition to the factors enumerated above, influences such as athletic or muscular build, physical activity and the general nutritive state cannot be ignored. In a racial study of metabolism the ideal method is to exclude all other potential influences on metabolism except race. The observations are to be confined to people of different races having more or less the same physical configuration and development. They must live as far as possible under the same conditions of temperature and humidity, engage themselves in the same degree of muscular activity and partake of the same quality and quantity of food. Rajagopal (*loc. cit.*) has found the metabolism in Indians to be 8 per cent below that of Europeans and he attributes this difference to a racial factor. In fact if due allowances could scientifically be made for the lower protein intake and for the other factors stated above the racial difference as such may to a large extent disappear. Wilson and

Roy (1938) do not favour the hypothesis of a racial factor being the cause of low B.M.R. of children investigated at Calcutta. The question of racial metabolism is so much complicated by the effects of previous diet, muscular activity, and environmental changes that the influence of the racial factor cannot be properly evaluated unless the above effects are, as far as possible, eliminated. It is proposed to make an extensive study of the racial factor in the future.

It will be interesting, however, to calculate on the basis of the results reported here the total caloric requirements of men and women in Bombay. The values given below are calculated for a man of average weight of 52·7 kg. and 166·1 cm. in height and for a woman with an average weight of 44·4 kg. and 152·8 cm. height, having a surface area of 1·57 and 1·37 sq. m., respectively.

		Calories.
Caloric requirement for man.		
Basal metabolism for 16 hours. $1·57 \times 34·5 \times 16$ ..	..	867
Basal metabolism during sleep 8 hours. $1·57 \times 34·5 \times 8 \times 0·9$		390
Allowance for moderate work		1,200
Allowance for specific dynamic action at 6 per cent of total metabolism.		147
	TOTAL ..	2,604
Caloric requirement for woman.		
Basal metabolism for 16 hours. $1·37 \times 32·05 \times 16$	..	708
Basal metabolism during sleep 8 hours. $1·37 \times 32·05 \times 8 \times 0·9$.		318
Allowance for household work		743
		1,769
Allowance for specific dynamic action at 6 per cent of total metabolism.		106
	TOTAL ..	1,875

SUMMARY.

The basal metabolism of 24 men and 52 women between the ages 18 and 35 years has been measured with the Sanborn's graphic metabolism tester. The basal metabolism of each subject was tested on three days with three observations on each day. The results are presented in three series ; the mean of the observations of the lowest two on the 2nd and 3rd day has been taken as the true basal metabolism of the subject. The means of the average oxygen consumption of the 2nd and 3rd series are 187 c.c. per minute for men and 152 c.c. for women. The value for heat production for men is 34·5 calories per sq. m. per hour and 32·05 calories per sq. m. per hour for women. The total caloric requirements of men and women per day are approximately 2,604 and 1,875 respectively.

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STUDIES IN HUMAN METABOLISM.

Part I.

PROTEIN METABOLISM IN INDIANS.

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THE determination of the protein requirements of man is an old controversial problem. The history of attempts to solve the problem of maintenance requirement has been reviewed by Martin and Robison (1922). As early as 1866, Voit showed that nitrogenous equilibrium could be maintained with an intake of 118 g. of protein equal to 0·27 g. N per kilo of body-weight per day. Later, various investigators including Hirschfeld (1887) and Sivén (1900) obtained minimum values varying between 0·08 g. and 0·18 g. N per kilo body-weight per day or 35 g. to 79 g. proteins daily for an adult weighing 70 kilos—most of the results being round about 0·1 g. N per kilo or 44 g. protein per 70 kilo body-weight. The investigations of Neumann (1902) and Chittenden (1905), undertaken to ascertain whether health and activity could be maintained over prolonged periods on a mixed diet of low content in protein, revealed that nitrogenous equilibrium was attained by Neumann on an intake of 0·15 g. N per kilo or 66 g. protein per 70 kilo body-weight and by Chittenden with 0·10 g. to 0·12 g. N per kilo or 44 g. to 53 g. protein per 70 kilo body-weight per day.

The whole question of the maintenance protein requirement in man was examined by Sherman (1920). He collected data from the literature and from his own experiments and applied a method of calculation commonly used in metabolism work. This is based on the assumption that the requirement is measured by the output near the equilibrium point at low intake: thus if, with any given

intake, there is a net loss of nitrogen from the body. intake is inadequate and the requirement is the sum of intake and net loss. If there is a net gain, the intake is in excess of the requirement for maintenance which is intake minus net gain. Sherman showed the requirement indicated by the general average of 109 experiments to be 0·635 g. protein per kilo of body-weight. or 44·4 g. per 'average man' of 70 kilos per day. Recently, Leitch and Duckworth (1937) have argued that the above assumption is unsound since the final result will depend on the range of intakes in the experiments and can be raised or lowered at will by extending or restricting the range. To remedy this difficulty they have adopted a clever method, which consists in fitting regression lines of output on intake to negative and positive balance data separately and calculating the point at which the chances of negative and positive balances are equal. Thus, the protein requirement for maintenance in adults (70 kilo body-weight) on a mixed diet including animal and plant proteins has been estimated by them to be about 50 g. daily. This estimate is in excellent agreement with the findings of Chittenden and also of Benedict *et al.* (1919) who found that his experimental subjects (University students) could maintain nitrogen equilibrium on an intake varying between 8 g. and 8·3 g. nitrogen or 50 g. and 52 g. protein per day. On the other hand Hindhede (1934, 1935) claims that equilibrium has been maintained on 32 g. 'digestible' protein from potato or whole meal bread. Dr. C. Rose is stated by Strieck (1937) to have attained nitrogen equilibrium on 24 g. to 29 g. of protein per day and to have remained in a state of excellent physical efficiency at this level. Terroine (1936) states that the maintenance requirement never exceeds 18·75 g. protein.

The importance of the protein element in nutrition in India has been stressed by Basu and his co-workers in investigation on biological value of proteins in this laboratory and also by Wilson *et al.* (1936). The problem of protein nutrition in India, however, has not been studied by any systematic metabolic studies conducted on Indian subjects. The object of the present investigation was to determine the nitrogen elimination of Indians on protein-free diets and also the actual nature of their protein requirements as indicated by studies of nitrogen balance on Indian subjects, and to find out if protein requirements are adequately covered by typical Indian dietaries. Rice and wheat are the two cereals which constitute the main items in the dietaries of Indian vegetarians. The diets investigated contained gradually increasing amounts of either of the cereals which constituted the main source of proteins. A practically constant amount of protein was supplied daily in the form of a fixed weight of a pulse (25 g.) and of vegetables (200 g.). Poor vegetarians in India derive their proteins mostly from rice or wheat and from pulses and vegetables, and milk scarcely figures in their dietaries. Parboiled milled rice and whole wheat (atta) were used in this investigation.

EXPERIMENTAL.

The experiments were conducted on two healthy young men, G. C. N. (19 years) and S. N. D. (24 years), weighing 49 kilograms each. They were kept under strict supervision and carried out ordinary duties in the laboratory. During the period that the nitrogen-free diet was being consumed, however, they did not do any laboratory work. They realized the importance of the investigations and co-operated whole-heartedly in the work.

A number of rice or whole wheat (atta) diets (exclusively vegetarian) composed of known amounts of foodstuffs as indicated in Table I were taken for a period of six consecutive days. The protein content of the diets was varied by varying the amount of rice or whole wheat (atta). The energy value of all the diets was kept constant by incorporating necessary quantity of nitrogen-free sago so that the sum of the weights of the cereal and sago was 600 g. in the case of G. C. N. and 500 g. in the case of S. N. D. Both with rice and wheat diets the experimental subjects were first given a diet containing the least amount of cereal. Diets containing gradually increasing amounts of cereal were then given and the intake of protein at which balance was actually or nearly obtained was discovered. Thus diet I, containing the lowest amount of cereal and hence of protein, was taken for six days, then without break diet II, and then without break diet III, all for six days. In some instances the experimental subject then returned to diet II, and then to diet I. In each series of experiments the cereal and the pulse were taken from the same stock every day and aliquots of vegetables taken daily were pooled for analysis. The experimental subjects were given carefully weighed diets which they consumed *in toto.*

For each diet which was taken for six days, urine and fæces excreted on the last three days only were quantitatively collected, the first three days being considered as a preliminary period to avoid any effect produced by the previous diet. Urine was collected quantitatively for 24 hours' periods and preserved over toluene to which some chloroform solution of thymol was added. The total volume of urine excreted each day was noted and then made up to a suitable volume for estimation. In order to be sure that the collection of urine was complete, the amount of creatinine eliminated daily was estimated and found to be uniformly constant. In the first series the fæces were collected and analysed daily but in other series of experiments the fæces were collected together for a period of three days since the daily dry weight of the fæces was found to vary considerably. Fæces were marked by carmine. These were preserved with a small quantity of glacial acetic acid, dried over a water-bath with frequent addition of alcohol, thoroughly powdered, weighed, and preserved in the refrigerator in stoppered bottles.

Total nitrogen was determined by Kjeldahl method.

RESULTS.

Data are arranged in Tables II to X. In Table II are indicated the minimum nitrogen outputs of two subjects on a protein-free diet. Tables III to X contain values for dietary nitrogen and urinary and fæcal nitrogen outputs on different rice and whole wheat (atta) diets, together with the digestibility and biological values of the mixed proteins of the corresponding diets. The biological value and digestibility have been calculated from the following formulæ :—

$$\text{Biological value} = 100 \times \frac{\text{Body nitrogen retained}}{\text{Food nitrogen absorbed}}$$

$$= 100 \left\{ 1 - \frac{\text{Total urine N} - \text{endogenous urine N}}{\text{Food N} - (\text{fæcal N} - \text{metabolic N})} \right\}$$

$$\text{Digestibility} = 100 \times \frac{\text{Food N} - (\text{fæcal N} - \text{metabolic N})}{\text{Food N}}$$

TABLE I.

Composition of typical diets (G. C. N.).

1. Protein-containing diet.

	Diet I.	Diet II.	Diet III.
Rice or whole wheat (atta)	150 g. or 200 g.	400 g.	600 g.
Sago	450 g. or 400 g.	200 g.	*Nil.*
Pulses (mung dhal, *Phaseolus mungo*)	25 g.	25 g.	25 g.
Fat (butter fat and mustard oil)	25 g.	25 g.	25 g.
Sugar	100 g.	100 g.	100 g.
Vegetables	200 g.	200 g.	200 g.
Common salt	5 g.	5 g.	5 g.
Lemon	one	one	one
Calorie content	3,000	3,000	3,000
Protein content—			
Rice diet	19·61 g. or 23·53 g.	39·22 g.	54·91 g.
Wheat diet	25·29 g. or 30·41 g.	52·75 g.	74·71 g.

In the case of S. N. D. identical diets were given except that the sum of the weights of cereal and sago was 500 g. so that with rice diets, diet I contained 250 g. rice and 250 g. sago, diet II 400 g. rice and 100 g. sago, while diet III contained 500 g. rice only. The exact amount of cereal taken is always indicated in the tables.

2. Protein-free diet.

	Diet.
Sago	650 g. (G. C. N.).
	550 g. (S. N. D.).
Sugar	150 g.

Composition of foodstuffs.

100 g. rice contained	..	1·075 g. to 1·263 g. N.
100 g. atta (whole wheat) contained	..	1·765 g. to 1·914 g. N.
100 g. pulse ,,	..	3·728 g. to 4·000 g. N.
100 g. vegetables ,	..	0·141 g. to 0·287 g. N.
100 g. sago ,,	..	0·008 g. N.

TABLE II.

Experiments with nitrogen-free diet, which was taken for six days while urine and fæces were collected during the last three days.

(1) Experimental subject—G. C. N.

Diet: 650 g. sago and 150 g. sugar.

Day of collec-tion.	Urine volume in c.c.	Urinary nitrogen (g.).	Fæcal weight (dry) (g.) per day.	Fæcal nitrogen (g.) per day.	Total nitrogen output (g) per day.
1st ..	1,790	1·459	..	..	..
2nd ..	1,595	1·934	..	..	..
3rd ..	1.390	1·104	..	..	..
Average ..	1,591	1·499	33·8	0·946	2·445

(2) Experimental subject—S. N. D.

Diet: 550 g. sago and 150 g. sugar.

Day of collec-tion.	Urine volume in c.c.	Urinary nitrogen (g.).	Fæcal weight (dry) (g.) per day.	Fæcal nitrogen (g.) per day.	Total nitrogen output (g.) per day.
1st ..	1,055	2·712	..	..	..
2nd ..	730	2·173	..	..	..
3rd ..	880	2·023	..	..	..
Average ..	888	2·302	17·88	1·133	3·435

TABLE III.

Nitrogen metabolism on diets containing different quantities of rice. (1st series).

Experimental subject—G. C. N.

Diet number.	Day of collection.	Total dietary nitrogen (g.) per day.	Urine volume in c.c.	Urinary nitrogen output (g.).	Faecal weight (dry) (g.).	Faecal nitrogen (g.).	Total nitrogen output (g.) per day.	Balance (g.) per day.	Biological value.	Digestibility.
I. 200 g. rice.	1st		1,785	2·299	35·95	1·366				
	2nd		2,223	2·152	32·15	2·025				
	3rd		2,430	2·916	44·65	2·007				
	Mean	3·765	2,146	2·455	37·58	1·799	4·254	−0·489	67	62
III. 600 g. rice.	1st		1,890	3·186	56·90	3·448				
	2nd		1,930	3·256	34·90	2·443				
	3rd		1,290	3·864	53·65	3·863				
	Mean	8·785	1,703	3·435	48·48	3·251	6·686	+2·099	70	67
II. 400 g. rice.	1st		2,855	2·282	27·60	1·766				
	2nd		1,850	2·407	28·90	2·023				
	3rd		1,535	2·789	51·60	3·457				
	Mean	6·275	2,080	2·492	36·03	2·415	4·907	+1·368	79	67
I. 200 g. rice.	1st		2,405	2·192	38·85	2·097				
	2nd		2,300	2·279	29·85	1·641				
	3rd		1,960	2·162	14·65	0·079				
	Mean	3·765	2,088	2·211	27·78	1·239	3·450	+0·315	79	74

TABLE IV.

Nitrogen metabolism on diets containing different quantities of whole wheat. (1st series).

Experimental subject—G. C. N.

Diet number.	Day of collection.	Total dietary nitrogen (g.).	Urine volume in c.c.	Urinary nitrogen (g.).	Fæcal weight (dry) (g.) per day.	Fæcal nitrogen output (g.) per day.	Total nitrogen (g.) per day.	Balance (g.).	Biological value.	Digestibility.
I. 150 g. atta	1st	..	1,400	2·140						
	2nd	..	1,710	2·614						
	3rd	..	2,990*	2·786						
	Mean	3·925	2,033	2·513	25·2	1·345	3·858	+0·067	71	72
II. 200 g. atta	1st	..	1,360	2·738						
	2nd	..	1,070	2·986						
	3rd	..	1,770	3·033						
	Mean	4·865	1,400	2·919	35·2	1·570	4·489	+0·376	67	73
III. 400 g. atta	1st	..	1,740	4·699						
	2nd	..	1,650	4·033						
	3rd	..	2,515	4·943						
	Mean	8·287	1,968	4·558	37·2	1·555	6·113	+2·174	60	83

* Rainy day.

TABLE V.

Nitrogen metabolism on rice diets. (2nd series).

Experimental subject—G. C. N.

Diet number.	Day of collection.	Total dietary nitrogen (g.) per day.	Urine volume in c.c.	Urinary nitrogen (g.).	Faecal weight (dry) (g.) per day.	Faecal nitrogen (g.) per day.	Total nitrogen output (g.) per day.	Balance (g.).	Biological value.	Digestibility.
I. 250 g. rice	1st	..	1,690	1·820						
	2nd	..	2,655	2·252						
	3rd	..	2,620	2·052						
	Mean	4·448	2,321	2·041	47·5	2·552	4·563	−0·115	81	53
II. 400 g. rice	1st	..	2,515	2·298						
	2nd	..	2,890	2·358						
	3rd	..	2,340	2·331						
	Mean	6·151	2,581	2·329	47·6	3·213	5·542	+0·609	77	55
III. 600 g. rice	1st	..	2,350	3·022						
	2nd	..	2,615	3·464						
	3rd	..	2,550	3·328						
	Mean	8·421	2,505	3·271	47·1	3·574	6·845	+1·576	69	62

III. 600 g. rice	1st	..	2,600	3·377						
	2nd	..	2,700	3·418						
	3rd	..	2,570	3·355						
	Mean	8·421	2,623	3·383	50·1	3·426	6·809	+1·621	68	63
II. 400 g. rice	1st	..	2,760	3·026						
	2nd	..	2,680	1·993						
	3rd	..	2,640	2·653						
	Mean	6·151	2,693	2·557	51·5	3·440	5·997	+0·154	71	52
I. 250 g. rice	1st	..	2,470	2·136						
	2nd	..	2,465	1·775						
	3rd	..	2,990	2·176						
	Mean	4·448	2,641	2·029	50·8	2·540	4·569	−0·121	81	53

TABLE VI.

Nitrogen metabolism on wheat (atta) diets. (2nd series).

Experimental subject—G. C. N.

Diet number.	Day of collection.	Total dietary nitrogen (g.) per day.	Urine volume in c.c.	Urinary nitrogen (g.).	Faecal weight (dry) (g.) per day.	Faecal nitrogen (g.) per day.	Total nitrogen output (g.) per day.	Balance (g.).	Biological value.	Digestibility.
I. 150 g. atta	1st	..	1,190	2·967						
	2nd	..	1,580	2·697						
	3rd	..	1,190	2·213						
	Mean	4·046	1,320	2·626	45	1·830	4·456	−0·410	64	63
II. 400 g. atta	1st	..	1,000	3·022						
	2nd	..	1,260	4·040						
	3rd	..	980	2·922						
	Mean	8·439	1,080	3·328	43·5	1·653	4·981	+3·458	76	82
III. 600 g. atta	1st	..	1,140	4·759						
	2nd	..	760	4·590						
	3rd	..	770	5·534						
	Mean	11·953	890	4·961	41·7	1·500	6·461	+5·492	70	88

III. 600 g. atta	1st	..	1,460	5·403						
	2nd	..	940	5·500						
	3rd	..	1,050	5·168						
	Mean	11·953	1,150	5·357	43·8	1·838	7·195	+4·758	70	86
II. 400 g. atta	1st	..	1,070	4·626						
	2nd	..	1,460	3·898						
	3rd	..	940	3·427						
	Mean	8·439	1,157	3·984	38·4	1·565	5·549	+2·890	68	83
I. 150 g. atta	1st	..	1,340	1·792						
	2nd	..	1,150	1·882						
	3rd	..	1,690	2·271						
	Mean	4·046	1,393	1·982	37·2	1·361	3·343	+0·703	87	73

TABLE VII.

Nitrogen metabolism on rice diets.

Experimental subject—S. N. D.

Diet number.	Day of collection.	Total dietary nitrogen (g.) per day.	Urine volume in c.c.	Urinary nitrogen (g.).	Fæcal weight (dry) (g.) per day.	Fæcal nitrogen (g.) per day.	Total nitrogen output (g.) per day.	Balance (g.).	Biological value.	Digestibility.
II. 400 g. rice	1st	..	1,260	3·282						
	2nd	..	1,370	2·915						
	3rd	..	850	2·816						
	Mean	5·858	1,160	3·004	36·2	2·592	5·596	+0·262	84	63
II. 400 g. rice	1st	..	1,660	3·339						
	2nd	..	2,320	3·528						
	3rd	..	2,140	3·210						
	Mean	5·858	2,040	3·359	42·2	3·063	6·422	—0·564	73	56
III. 500 g. rice	1st	..	2,170	3·137						
	2nd	..	2,480	3·112						
	3rd	..	2,420	3·085						
	Mean	6·986	2,356	3·112	46·2	3·205	6·317	+0·669	84	61

TABLE VIII.

Nitrogen metabolism on whole wheat (atta) diets.

Experimental subject—S. N. D.

Diet number.	Day of collection.	Total dietary nitrogen (g.) per day.	Urine volume in c.c.	Urinary nitrogen (g.).	Fæcal weight (dry) (g.) per day.	Fæcal nitrogen (g.) per day.	Total nitrogen output (g.) per day.	Balance (g.).	Biological value.	Digestibility.
I. 300 g. atta	1st	..	950	4·864						
	2nd	..	880	4·685						
	3rd	..	620	4·204						
	Mean	7·064	816	4·584	47·9	2·146	6·730	+0·334	62	74
II. 400 g. atta	1st	..	620	6·142						
	2nd	..	630	5·168						
	3rd	..	640	4·787						
	Mean	8·970	630	5·365	46·65	2·283	7·648	+1·322	62	77
III. 500 g. atta	1st	..	580	5·940						
	2nd	..	610	6·489						
	3rd	..	760	7·427						
	Mean	10·876	650	6·618	62·5	2·761	9·379	+1·497	53	77

In Table IX the intakes at which nitrogen balance was attained by the experimental subjects have been collected together and the mean protein requirement for maintenance has been noted :—

TABLE IX.

Maintenance protein requirement.

Experimental subject.	RICE DIET.			Experimental subject.	ATTA DIET.		
	Nitrogen requirement (g.).	Mean protein requirement (g.).	Protein requirement per 70 kilo body-weight (g.).		Nitrogen requirement (g.).	Mean protein requirement (g.).	Protein requirement per 70 kilo body-weight (g.).
G. C. N. (49 kilo)	4·254	26·3	37·5	G. C. N.	3·858	24·3	34·7
	3·450				4·456		
	4·563						
	4·569				3·343		
Mean	4·209			Mean	3·885		
S. N. D. (49 kilo)	5·596	37·6	53·6	S. N. D.	6·730	42	60
	6·422						
Mean	6·009						

TABLE X.

Biological value and digestibility of mixed proteins.

Experimental subject.	RICE DIETS.			Experimental subject.	WHEAT DIETS.		
	Level of nitrogen intake (g.) per day.	Biological value.	Digestibility.		Level of nitrogen intake (g.) per day.	Biological value.	Digestibility.
G. C. N. ..	3·765	67	62	G. C. N. ..	3·925	71	72
,, ..	3·765	79	74	,, ..	4·046	64	63
,, ..	4·448	81	53	,, ..	4·046	87	73
,, ..	4·448	81	53	,, ..	4·865	67	73
S. N. D. ..	5·858	84	63	S. N. D. ..	7·064	62	74
,, ..	5·858	73	56	G. C. N. ..	8·287	60	83
G. C. N. ..	6·151	77	55	,, ..	8·439	76	82
,, ..	6·151	71	52	,, ..	8·439	68	83
,, ..	6·275	79	67	S. N. D. ..	8·970	62	77
S. N. D. ..	6·986	84	61	,, ..	10·876	53	77
G. C. N. ..	7·788	66	67	G. C. N. ..	11·953	70	88
,, ..	7·788	70	67	,, ..	11·953	70	86
,, ..	7·788	77	73	,, ..	12·117	60	81
,, ..	8·421	69	62	,, ..	12·117	61	82
,, ..	8·421	68	63	..	..	..	..
,, ..	8·785	70	67	..	..	..	..
Mean ..	6·418	75	62	..	8·364	66·5	78

Some experiments were carried out to discover if the retention of nitrogen may be augmented by the addition of sugar to diets of adequate energy value and containing more than enough protein for maintenance. In these experiments the composition of the diets was the same as diet III in Table I except that increasing amounts of sugar were added.

TABLE XI.

Effect of increase in intake of sugar on protein retention (rice diet).

Experimental subject—G. C. N.

Diet number.	Day of collection.	Total dietary nitrogen (g.) per day.	Urine volume in c.c.	Urinary nitrogen (g.).	Faecal weight (dry) (g.) per day.	Faecal nitrogen (g.) per day.	Total nitrogen output (g.) per day.	Balance (g.).	Biological value.	Digestibility.
Calories—3,000. Diet III.	1st	..	2,650	3·400						
	2nd	..	2,710	3·335						
	3rd	..	2,385	3·758						
	Average ..	7·788	2,582	3·497	44·8	2·858	6·355	+1·433	66	67
Calories—3,500. Diet III + 125 g. sugar.	1st	..	1,960	3·401						
	2nd	..	2,070	3·262						
	3rd	..	2,205	3·126						
	Average ..	7·788	2,078	3·263	46·0	2·902	6·165	+1·629	70	67
Calories—4,000. Diet III + 250 g. sugar.	1st	..	2,160	3·079						
	2nd	..	1,415	2·877						
	3rd	..	1,840	2·870						
	Average ..	7·788	1,805	2·942	40·4	2·379	5·321	+2·467	77	73

TABLE XII.

Effect of increase in intake of sugar on protein retention (whole wheat diet).

Experimental subject—G. C. N.

Diet number.	Day of collection.	Total dietary nitrogen (g.) per day.	Urine volume in c.c.	Urinary nitrogen (g.).	Faecal weight (dry) (g.) per day.	Faecal nitrogen (g.) per day.	Total nitrogen output (g.) per day.	Balance (g.).	Biological value.	Digestibility.
Calories—3,000. Diet III.	1st	..	1,400	6·226						
	2nd	..	1,640	5·572						
	3rd	..	1,470	5·372						
	Average	12·117	1,503	5·723	49·3	2·402	8·125	+3·992	60	81
Calories—3,500. Diet III + 125 g. sugar.	1st	..	1,060	6·982						
	2nd	..	1,160	5·113						
	3rd	..	1,000	4·848						
	Average	12·117	1,073	5·647	46·4	2·382	8·029	+4·088	61	82

DISCUSSION.

(a) *The minimum nitrogen expenditure.*—This means the absolute minimum total nitrogen excretion in the urine and fæces, when a normal adult is maintained on a practically nitrogen-free diet with calorie requirements supplied in excess by carbohydrate and fat. The literature of this subject has been reviewed by Smith (1926). In the present case the total urinary nitrogen excretions were 1·499 g. or 0·030 g. per kilo (G. C. N.) and 2·302 g. or 0·047 g. per kilo (S. N. D.). If figures for fæcal nitrogen outputs are added, the total nitrogen eliminations become 0·05 g. (G. C. N.) and 0·07 g. (S. N. D.) per kilo body-weight. In other words, the absolute minimum amount of protein, capable of quantitatively replacing the 'wear and tear' quota of the human body *in toto*, can be theoretically calculated as 22 g. and 30 g. respectively with a mean value of 26 g. These values lie remarkably close to the values of Terroine (*loc. cit.*) and Hindhede (*loc. cit.*).

(b) *The minimum requirement of protein for maintenance.*—The minimum amount of protein that is just necessary to keep an adult in nitrogen balance is known as the maintenance requirement of protein. It could be expected that just as basal heat production is a measure of the minimum energy requirement, the amount of nitrogen excreted by an adult on a protein-free diet, when fed in the form of protein, should be able to maintain him in nitrogen balance. In practice, however, it is not quite so. The minimum amount of protein that is just necessary to keep the nitrogen balance of an adult is always greater than his minimum nitrogen expenditure. The digestibility of proteins, as also the assortment of amino-acids in the digested proteins, are two of the factors which are responsible for this. Our experiments to determine the maintenance protein requirement of subjects consuming Indian vegetarian diets involved adherence to an otherwise uniform diet with periodical increase or decrease of protein consumption, without alternation of the energy intake, until it was found that the subject could maintain himself in nitrogen equilibrium on a certain amount of protein of the kinds which the diet in question furnished. The experimental conditions were thus almost ideal and our results are free from the uncertainties which are associated with the graphical method of calculation. Thirty metabolism experiments were performed and the protein output in ten experiments in which protein balance was nearly obtained are noted in Table IX and show that the maintenance protein requirement of an adult of 70 kilo body-weight is 37·5 g. (G. C. N.) and 53·6 g. (S. N. D.) protein on rice diet and 34·7 g. (G. C. N.) and 60 g. (S. N. D.) on atta diet. In the 109 experiments reported by Sherman (*loc. cit.*) protein requirements per 70 kilos of body-weight varied between 21 g. and 65 g. with an average of 44·4 g. We have obtained protein balance in ten experiments on two individuals and shall be hardly justified taking an average of the two sets of figures. However, the average of protein requirement values obtained by us is 46·4 g. protein and is in excellent agreement with the Sherman value of 44·4 g. protein but is lower than the Leitch and Duckworth (*loc. cit.*) value of 50 g. obtained by graphical method. Our observations show that a vegetarian rice diet (600 g. rice), typical of that consumed by Indians of the poorer classes and containing about 54 g. of protein, is sufficient to maintain an adult in equilibrium but does not contain the 50 per cent margin which has been advocated by Sherman. The typical wheat diet (600 g. wheat), on the other hand, which contained about 74 g. of protein, reaches the Sherman standard in this respect.

(c) *Protein retention.*—Balance studies (Tables III to X) show that, at any level of protein intake, the rice diet effected better retention of nitrogen than the atta diet but at any level of food intake, i.e., weight for weight, the reverse held good. On two typical vegetarian diets, containing 600 g. of rice or atta, G. C. N. retained about 1·5 g. and 5·1 g. nitrogen respectively. S. N. D., on 500 g. rice or atta diet, retained 0·67 g. and 1·5 g. nitrogen respectively. This clearly establishes the superiority of atta over rice in typical dietaries containing equal weights of the two cereals in respect of capacities to fulfil the protein requirements of the body.

(d) *Digestibility and biological value of mixed protein.*—The percentage digestibility of rice in Indian dietaries was studied by McCay (1910). He showed that the percentage absorption of food nitrogen with a diet containing 25 oz. rice and 4 oz. dhal was only 52 per cent, while at higher level of rice intake it was still lower, approximately 45 per cent. The results on the digestibility of rice diets obtained in the present investigation (Table X) are in general agreement with those of McCay. The average values of the coefficient of digestibility of the mixed proteins in rice and atta diets have been found to be 62 and 78 respectively. The lower values obtained by McCay are due to his not taking the metabolic nitrogen of the fæces, i.e., nitrogen excreted in the fæces on a nitrogen-free diet, into consideration in calculating the digestibilities. The digestibility of rice proteins obtained by experiment on human subjects in this investigation is lower than the values obtained with rats by Basu and Basak (1937) in this laboratory. It is important to determine the biological values of actual dietaries rather than the values of individual foodstuffs, since there is the probability that the proteins of the individual ingredients in a diet may supplement each other. Supplementary relations between proteins of different foodstuffs have already been studied in rat experiments reported from this laboratory (Basu and Basak, *loc. cit.*; Basu and De, 1938). Results obtained with rats may or may not be applicable to human beings and hence determination of the biological values of proteins by experiments on human subjects is desirable.

Basu and Basak (*loc. cit.*) have previously found the biological value of rice to be 80 at 5 per cent level of intake in rat experiments. In the present investigation the average biological value of the mixed protein of rice, pulse (mung dhal, *Phaseolus mungo*) and vegetables at an average level of 5 per cent intake has been found to be 75 and that of whole wheat, pulse, and vegetables at similar level to be 66·5. The authors are well aware, however, that although a rough comparison is legitimate, the biological value of the mixed proteins contained in the diets used in these experiments and the figures obtained with individual foodstuffs in other investigations are not strictly comparable.

(e) *Effect of ingestion of excess calories.*—It is known that available carbohydrates and also fats (although to a less extent) spare protein and consequently facilitate its retention (*cf.* Larson and Chaikoff, 1937; Cuthbertson and Munro, 1937). Some experiments were conducted with rice and with atta diet (600 g.), the total calorie intake being gradually increased from 3,000 to 4,000 by the addition of sugar. Results are grouped in Tables XI and XII. It is found that sugar, even when added to an already adequate diet, helps retention of protein.

SUMMARY.

1. The minimum nitrogen excretion of two adults (both of 49 kilo body-weight) on a protein-free diet was determined and found to be 1·499 g. (G. C. N.) and 2·302 g. (S. N. D.) in the urine and 0·946 g. (G. C. N.) and 1·133 g. (S. N. D.) in the fæces. The total outputs have been found to be 0·05 g. N (G. C. N.) and 0·07 g. N (S. N. D.) per kilo body-weight.

2. The average minimum protein requirements for maintenance per 70 kilo body-weight on rice diets, containing rice, pulse, and vegetables, were 37·5 g. (G. C. N.) and 53·6 g. (S. N. D.) and on wheat diet containing whole wheat, pulse, and vegetables 34·7 g. (G. C. N.) and 60 g. (S. N. D.). Out of thirty metabolism experiments the average protein requirement from ten experiments in which nitrogen balance was nearly obtained was found to be 46·4 g. protein per 70 kilo body-weight. Typical rice or wheat diets resembling those consumed by poor Indians without any milk maintain adults in nitrogen balance. The typical rice diet does not, while the wheat diet does, contain the allowance of 50 per cent above maintenance level advocated by Sherman.

3. The average values for the digestibilities of mixed proteins of rice and whole wheat diets were found to be 62 and 78 respectively and the mean biological values 75 and 66·5 respectively.

4. The retention of protein was greater on a diet largely composed of whole wheat than on a diet of equal calorie value containing similar quantities of rice.

5. Sugar when added to a diet already adequate in energy value spares protein and effects better nitrogen retention.

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VITAMIN-C CONTENT OF THE CHILLIES, ONION, AND GARLIC, BOTH IN THE RAW STATE AND WHEN BOILED WITH WATER.

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OF all the known vitamins, vitamin C is the most sensitive to heat. In the pure state it is extremely heat-labile, but when it occurs in natural products it can resist the destructive action of heat to a considerable extent. Recently, an apparent increase of vitamin C on heating has been observed in the case of the cabbage (Ahmad, 1935), potato (Levy, 1937), and peas (Oliver, 1936). Three different views have been put forward to explain this anomaly. McHenry and Graham (1935) hold the view, that in the natural products vitamin C occurs as an ester-like substance and on the application of heat this ester is hydrolysed and the ascorbic acid is set free. Mack and Tressler (1937) suggest that the apparent increase is due to the inactivation of the enzyme present in the natural substance. Oliver (*loc. cit.*) on the other hand, while inclining to the ' ester theory ', accounts for the enhanced value of this vitamin to a more complete extraction of the cooked materials than the raw one, owing to the softening of woody tissues. Though the ester theory has been supported by a number of workers yet the explanation offered by Oliver seems to be a very cogent one. The primary aim of the present paper is to show the effect of boiling with water on the vitamin-C content of a few common auxiliaries of our table, one of which shows a remarkably high increase of vitamin C in the process.

ONION, ONION SCAPE, AND GARLIC.

Several varieties of the stuffs were taken, freed from the outer dried pulp or skin, and cut into pieces. Ten grammes of each of the substances were weighed out

into a mortar with 2·5 c.c. of 20 per cent trichlor-acetic acid and a few grammes of Merck's sea sand. They were then finely ground with the addition of a few c.c. of distilled water and centrifuged. The centrifugate was made up to 100 c.c. and titrated against 2 : 6-dichlorophenol-indophenol according to the method of Harris and Ray (1933) as modified by Ghosh and Guha (1935) and Guha and Ghosh (1935). In the case of pigmented extracts the method of Tillmans *et al.* (1932) as modified by McHenry and Graham (*loc. cit.*) was adopted. Three sets of experiments were made from stuffs procured from the market on different dates, so as to obtain a good average. The results obtained are given in Table I. Another lot of 10 g. of each of the substances were taken in a basin and boiled in each case with 150 c.c. of distilled water for 15 minutes, the added water being just evaporated by the time. They were then taken in a mortar with 2·5 c.c. of 20 per cent trichlor-acetic acid solution and a few grammes of Merck's sea sand and treated in the aforesaid manner. The findings are given in Table II.

TABLE I.

Ascorbic acid in mg. per 100 g.

Onion (*Allium cepa*), big variety.	Onion, medium variety.	Onion, small variety.	Onion scape.	Garlic (*Allium sativum*).
8·33	6·67	5·56	8·33	6·49
9·09	7·14	7·14	14·29	7·46
8·33	7·94	7·14	9·09	7·14
AVERAGE 8·58	7·25	6·61	10·57	7·03

TABLE II.

Ascorbic acid in mg. per 100 g.

Onion (*Allium cepa*), big variety.	Onion, medium variety.	Onion, small variety.	Onion scape.	Garlic (*Allium sativum*).
2·94	2·50	1·79	4·00	3·70
3·85	2·00	1·85	8·33	2·63
2·27	2·94	2·00	6·25	3·33
AVERAGE 3·02	2·48	1·88	6·19	3·22

CHILLIES.

Both green and ripe chillies of the several varieties investigated were procured fresh from the market each day.   The stalks as well as the dust particles adhering to the individual chillies were carefully removed.   Ten grammes were then weighed out in a mortar with 2·5 c.c. trichlor-acetic acid and a few grammes of Merck's sea sand.   They were then treated in the usual way.   Another 10 g. were then taken out and the experiments were repeated by boiling exactly as in the case of the onion. The values obtained from the raw and the cooked stuffs are given in Table III and Table IV respectively :—

TABLE III.

Ascorbic acid in mg. per 100 g.

UNRIPE.			RIPE.		
Chillies (*Capsicum*), big variety.	Chillies, small variety.	Chillies, Patnai variety.	Chillies, big variety.	Chillies, small variety.	Chillies, Patnai variety.
20·00	9·09	90·09	142·85	166·67	181·82
28·57	10·00	71·43	200·00	166·67	166·67
9·52	10·64	80·00	181·82	83·33	166·67
AVERAGE 19·36	9·91	80·51	174·89	138·89	171·72

TABLE IV.

Ascorbic acid in mg. per 100 g.

UNRIPE.			RIPE.		
Chillies (*Capsicum*), big variety.	Chillies, small variety.	Chillies, Patnai variety.	Chillies, big variety.	Chillies, small variety.	Chillies, Patnai variety.
153·85	100·00	125·00	200·00	153·85	250·00
142·85	125·00	166·67	250·00	166·67	222·22
153·85	90·91	166·67	222·22	153·85	181·82
AVERAGE 150·18	105·30	152·78	224·07	158·12	218·01

From Tables I and II it is evident that onion, onion scape, and garlic lose their vitamin C in the process of cooking. The approximate losses sustained by the above materials on boiling with water are 65, 40, and 55 per cent, respectively.

Tables III and IV show that a very small quantity of vitamin C is present in the green stuff, while in the ripe one from 2 to 14 times increase is noticed and nearly the same increase is observed when a green sample is boiled with water. In the former case the combined or 'ester' form of ascorbic acid becomes free by the action of the enzyme during the process of ripening, while in the latter case the breaking down of the ester form is effected possibly by the action of heat and also to some extent by the softening of the pulps in the process of boiling the stuffs with water. The ripe chillies are found to give only about 30 per cent increase of the vitamin-C content on cooking which may be accounted for mostly by the complete extraction of it from the softening of the pulps. It is also noticed that big onions are richer in vitamin C than the small ones and onion scape, which is largely consumed in winter, is not a negligible source of the vitamin.

ACKNOWLEDGMENT.

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THE DEFECTS OF TAPIOCA AS A STAPLE FOOD.

BY

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TAPIOCA is grown and consumed in Travancore in south-west India. The population of Travancore State is some 6 millions and its total area some 7,625 square miles or roughly 5 million acres. About 2 million acres are under cultivation and of these about 400,000 acres are devoted to growing tapioca. According to an estimate kindly supplied by the Director of Agriculture and Fisheries, Travancore, about 75 per cent of the population consumes tapioca in various forms. In many parts of the State it forms the main ingredient in the diet of the poorer classes.

Karunakaran (1938), in a report to the Technical Commission on Nutrition of the League of Nations, makes the following statement :—

'One striking change that has occurred in the diet of the people of Travancore during the last two or three decades is the general use of tapioca as a staple food, particularly among the poorer classes. . . . Lands which 20 or 30 years ago were used for growing horse gram and other pulses are now used for the cultivation of tapioca. There can be no doubt that this change in agricultural policy is the result of pressure on land. There is no other tuber or cereal which, per unit area, can give as many calories as tapioca. It has been estimated that 0·76 acre under potato will supply the calorie requirements of an adult man for one year, while to obtain the same energy value from wheat 1·45 acre, and for milk 3·95 acres, will be necessary. In the case of tapioca 0·2–0·4 acre alone is necessary, the average yield of tapioca ranging from 2 to 5 tons per acre. This, in all probability, is the reason for the extensive cultivation and use of this food. Tapioca has, undoubtedly, helped the people to escape famine and the privations which have often affected other parts of India ; but it has, at the same time, accounted for a progressive deterioration in their physique and an increased incidence of diseases of various kinds '.

It was observed by Krishnan (1939) that children living in a tapioca-producing area in Travancore were smaller and lighter at all ages than rice-eating children elsewhere in South India. There was no apparent difference in race to account for the poorer physique of the tapioca-eaters, and this was ascribed to the consumption

of a staple which is defective in various respects.   Tapioca, being a root and not a cereal, contains only 0·5 to 2·0 per cent of protein, and in addition it is deficient in various vitamins and mineral salts.   The following analysis of tapioca is taken from Health Bulletin No. 23 (1938) :—

	Grammes per 100 grammes.		Grammes per 100 grammes.
Moisture ..	.. 59·40	Carbohydrate (by difference).	39·00
Protein ..	.. 0·70	Calcium ..	.. 0·05
Fat (ether extractives) ..	0·20	Phosphorus ..	.. 0·02
Mineral matter	.. 1·00	Carotene ..	.. *Nil*

Other investigators have given a slightly higher figure for protein content.

Experimental.

The nutritive value of tapioca as a staple is thus a question of practical importance.   In the present investigation we have studied the problem using the rat-growth method previously employed to investigate the value of the ' poor South Indian diet ', which is largely composed of rice, and the effect of adding various supplements to the diet (Aykroyd and Krishnan, 1937*a*, *b*).   The basal diet used in the present experiments was similar to the ' poor South Indian diet ' except that rice was replaced by tapioca.   Its composition was as follows :—

	Oz.	Grammes.
Tapioca (*Manihot utilissima*)	21·00	596·0
Dhal arhar (*Cajanus indicus*)	0·70	20·0
Black gram (*Phaseolus mungo*)	0·70	20·0
Brinjal (*Solanum melongena*)	1·00	28·0
Amaranth leaves (*Amaranthus gangeticus*) ..	0·50	14·0
Raw plantain (*Musa paradisiaca*)	0·50	14·0
Gingelly oil (*Sesamum indicum*)	0·10	3·0
Coco-nut (*Cocos nucifera*)	0·05	1·4
Meat (mutton)	0·06	1·7

The quantities as stated correspond roughly to human adult daily intake.   The quantities of food other than tapioca included in the diet are roughly equivalent to the quantities of these foods consumed by poor rice-eaters in South India, as discovered by diet surveys.   The tapioca was obtained from Travancore.

Groups of 12 young rats, about 50 to 55 grammes in weight, were given the above diet, mixed and fed in the proportions indicated, for a period of 10 weeks. Each group contained equal numbers of males and females.   Supplements of various kinds were added to the diet and their effect observed.   These included casein, soya bean, green gram (*Phaseolus radiatus*), Bengal gram (*Cicer arietinum*), horse gram (*Dolichos biflorus*), dried yeast, cod-liver oil, skimmed milk, and calcium lactate. The quantities of the supplements included in the basal diet (with the exception of calcium lactate) corresponded with additions which might feasibly be made to human diets and are stated in grammes and ounces.

The results of these experiments are given in the Table and the growth curves of certain groups are shown in the Chart :—

TABLE.

The results of feeding young rats on a tapioca diet and the same diet variously supplemented.

Diet.	Average weekly increase in weight (g.).	REMARKS.
Tapioca diet alone	..	All animals died within 10 weeks.
„ „ + 2 drops cod-liver oil daily per rat ..	..	do.
Supplements incorporated in the diet.		
„ „ + 0·25 oz. (7 g.) calcium lactate	..	do.
„ „ + 0·5 oz. (14 g.) dried yeast	..	Five died.
„ „ + 1·5 oz. (42 g.) casein	5·9	All survived.
„ „ + 1·5 oz. (42 g.) casein + 0·25 oz. (7 g.) calcium lactate.	6·5	do.
„ „ + 1·5 oz. (42 g.) casein + 0·25 oz. (7 g.) calcium lactate + 0·5 oz. (14 g.) dried yeast.	6·2	do.
„ „ + 1·5 oz. (42 g.) skimmed milk powder ..	4·4	do.
„ „ + 0·64 oz. (18 g.) casein	2·7	One death.
„ „ + 3·5 oz. (100 g.) soya bean	1·5	No deaths.
„ „ + 1·5 oz. (42 g.) soya bean	..	7 deaths.
„ „ + 2·6 oz. (73 g.) green gram (*Phaseolus radiatus*).	0·8	2 deaths.
„ „ + 3·8 oz. (108 g.) Bengal gram (*Cicer arietinum*).	1·7	do.
„ „ + 2·9 oz. (82 g.) horse gram (*Dolichos biflorus*).	..	All died.

CHART.
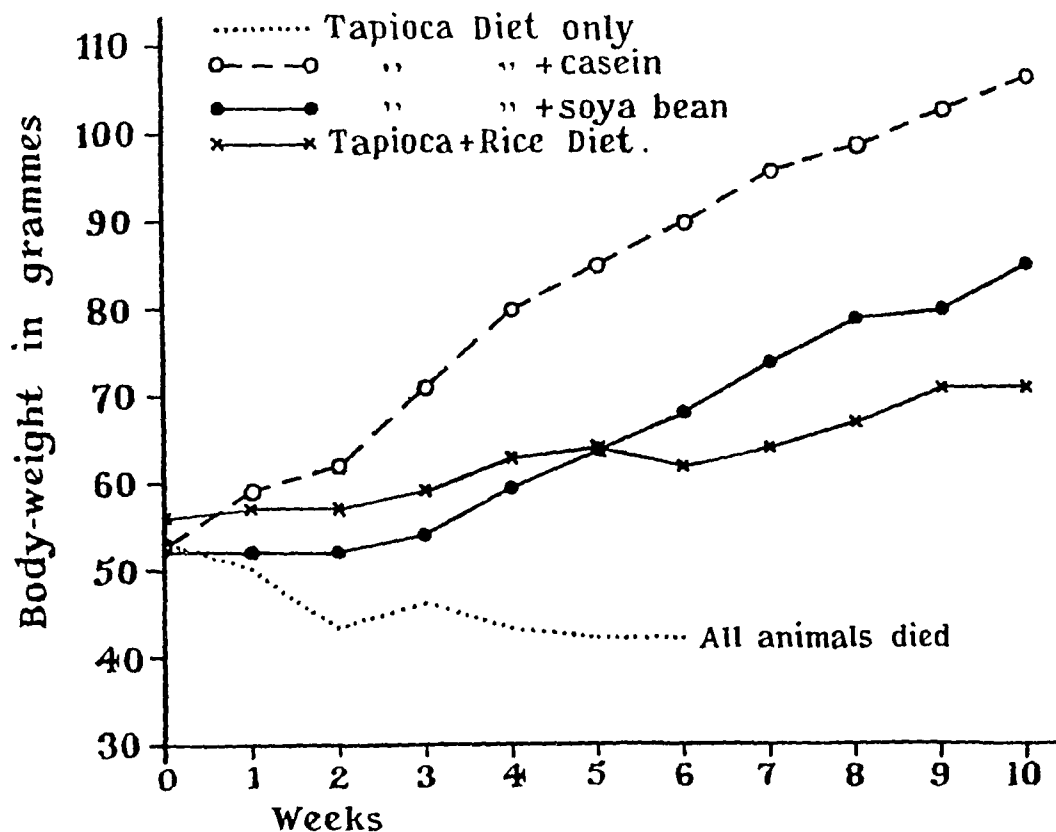
Weekly increase in weight on the tapioca diet, etc.

The addition of cod-liver oil and calcium lactate had little effect, and with the yeast supplement 5 of the animals died and the remainder failed to grow. The addition of a pure protein (casein from milk) had, however, a marked effect. The animals survived and the weekly increase in body-weight was in the neighbourhood of 6 grammes. Similarly, skimmed milk, which contains both casein and lactalbumen, was of definite supplementary value.

An attempt was made to compare the effect of pulse protein with that of casein. The addition of 3·5 oz. of soya bean provided as much protein as 1·5 oz. of casein; while, however, this supplement enabled the animals to survive, growth (1·5 g. per week) was very poor. The supplements of 1·5 oz. of soya bean, 2·6 oz. of green gram, 3·8 oz. of Bengal gram, 2·9 oz. of horse gram, and 0·64 oz. of casein supplied roughly equivalent amounts of protein. None of the pulse supplements, given in these quantities, was effective. Seven animals in the group receiving this proportion of soya bean died and all the animals died in the horse-gram group. The green-gram and Bengal-gram groups showed a higher survival rate but little increase in weight occurred. Presumably the relatively low biological value of the proteins of these pulses (Swaminathan, 1938) partially accounts for their failure to act as effective

supplements to the tapioca diet in the quantities given. A similar quantity of animal protein (0·64 oz. casein) had a better effect.

Tapioca and rice diet.—While these experiments were in progress a diet survey was carried out in a tapioca-eating area in Travancore (Krishnan, *loc. cit.*). A group of 9 families was investigated. The diet of these families differed from the 'tapioca diet' described above in that it contained a fair proportion of rice and also of yams. Its average composition (per adult man per day) was approximately as follows :—

Tapioca and rice diet.

	Oz.	Grammes.
Tapioca (*Manihot utilissima*)	18·00	509·0
Milled parboiled rice	9·00	255·0
Dhal arhar (*Cajanus indicus*)	1·00	28·0
Brinjal (*Solanum melongena*)	1·00	28·0
Amaranth leaves (*Amaranthus gangeticus*) ..	0·05	1·4
Yams (*Amorphophallus campanulatus*)	5·00	140·0
Banana (green) (*Musa sapientum*)	1·00	28·0
Milk	0·50	14·0
Meat	0·10	3·0
Fish	0·60	17·0
Coco-nut oil	0·25	7·0

This diet was fed to a group of young rats. All survived, average weekly increase in weight being 3·3 g. The addition of 1·5 oz. of casein, however, enhanced the nutritive value of this diet for rats ; with this supplement the weekly increment was 5·8 g.

DISCUSSION.

In previous papers (Aykroyd and Krishnan, 1937*a*, *b*) we pointed out that rat-growth experiments of this nature give only an *indication* of the nutritive value of human diets and of the additions to such diets which are likely to prove most valuable. 'They do, however, give us a line to follow in the difficult task of discovering methods of improving the South Indian diet consistent with economic realities'. The present experiments should be viewed in this light. It may also be recalled that the young rats used for the growth experiments are well stored with vitamin A at the outset, so that the vitamin-A content of the basal diet and the supplements have little effect on the experimental results. It was previously recorded that the animals given the tapioca diet supplemented with cod-liver oil all died.

Young rats, fed on a tapioca diet containing other foods in quantities corresponding to those contained in typical South Indian diets, die in a few weeks. While it does not necessarily follow that human beings cannot survive on such a diet, the experiment does suggest that tapioca has great disadvantages as a staple food, and points a warning. Broadly, the fact emerges that the main deficiency of the tapioca diet is in the quantity and probably the quality of protein which it contains. The low protein content of tapioca is an ascertained fact. The biological value of tapioca proteins has not, however, as yet been investigated.

The addition of yeast and calcium lactate had little effect. In the calcium lactate group all died; in the yeast group about half the animals survived for 10 weeks but did not grow. The failure of the tapioca diet to support life cannot therefore be explained as a result of a lack of the vitamin-B complex or calcium. Similar additions of yeast and calcium lactate improved the nutritive value for rats of the 'poor South Indian diet', largely based on rice (Aykroyd and Krishnan, 1937b).

The supplements of casein and skimmed milk powder, on the other hand, improved the basal diet and enabled the animals to survive and grow a little. The addition of yeast and calcium lactate to the casein-supplemented diet did not produce any further enhancement of the growth rate; it is probable that in these experiments insufficiency of first class protein remained the limiting factor in growth. Vegetable proteins contained in pulses are less effective supplements than milk proteins.

The tapioca and rice diet, based on the result of an actual diet survey, was of higher nutritive value. Additional protein is provided by rice, to some extent by the small quantities of milk, fish, and meat included, and to a very small extent by vegetables. The proteins of rice are of relatively high biological value (Swaminathan, *loc. cit.*). Nevertheless the diet is deficient in protein, as is shown by the supplementary effect of casein. There are no means at present of knowing how far this diet is typical of that consumed in the tapioca-eating areas of Travancore. It appears, however, that the consumption of tapioca is increasing in this part of India, and the tendency is in the direction of a diet containing more tapioca and less rice and other foods. The present experiments suggest that this change, which may be largely due, as Karunakaran (*loc. cit.*) suggests, to population pressure, is a most undesirable one, which will have a deleterious effect on the health of the people. The defects of a diet largely based on tapioca should be realized by the public health and agricultural authorities concerned, and an attempt made to check or reverse the tendency. In tapioca-eating areas, there is a special need for foods rich in proteins of high biological value, such as milk, fish, or meat. Policies for improving diet require a somewhat different orientation in tapioca-eating and rice-eating areas. In the former the emphasis should be on the protein factor. The main deficiency of the poor rice-eater's diet, on the other hand, is not in protein, but rather in certain vitamins, notably various elements in the B complex, and calcium.

SUMMARY.

1. Young rats fed on a diet largely composed of tapioca, containing in addition pulses, vegetables, etc., in amounts corresponding to the usual consumption of poor rice-eaters in South India, die within a few weeks.

2. The addition of casein or skimmed milk to this diet permits survival and an average weekly increase in body-weight of about 6 g. for 10 weeks.

3. A supplement of soya bean, given in such quantities that the addition of protein was equivalent to that supplied by a supplement of 1·5 oz. casein, was less effective, weekly increase in weight being only 1·5 g. Supplements of soya bean and other pulses containing about half this amount of protein produced little growth and deaths occurred in these experimental groups.

4. Cod-liver oil and calcium lactate had no effect on the survival rate. On the basal diet supplemented by yeast 5 out of 12 animals died.

5. Animals fed on a tapioca and rice diet based on the results of a diet survey survived, but growth was poor. The diet was improved by the addition of casein.

6. It is concluded that tapioca as a staple is unsatisfactory because of a deficiency in the quantity, and possibly the quality, of its protein. The present tendency, in certain parts of India for the consumption of tapioca to increase at the expense of that of other foods including rice, is undesirable from the standpoint of nutrition.

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THE INFLUENCE OF VARYING LEVELS OF CALCIUM INTAKE ON THE BIOLOGICAL VALUE OF PROTEINS.

BY

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It has been shown in this Laboratory that the nutritive value of the 'poor South Indian diet' for rats is increased by the addition of a calcium salt, and this result has been confirmed by experiments on school children (Aykroyd and Krishnan, 1937, 1938). The 'poor South Indian diet' is composed largely of rice, a cereal deficient in calcium. The author (Swaminathan, 1937*a*, *b*, *c*, *d*) has shown that, while the proteins of mixtures of rice and different pulses are of fairly high biological value, the addition of small quantities of skimmed milk powder to such mixtures increases the biological value of the proteins. Milk, in addition to containing first class protein, is rich in calcium. Toepfer and Sherman (1936) observed that 'increase in the calcium or calcium and phosphorus content of an already adequate diet resulted in a more rapid and more efficient growth with greater economy in the use of food'.

Conceivably, the effect of calcium in supplementing the 'poor South Indian diet' might be to some extent due to an improvement in the biological value of the proteins contained in the diet, resulting from better assimilation and utilization. To test this possibility, an experiment has been carried out in which the biological value of a mixture of casein and rice protein was determined at varying levels of calcium intake.

EXPERIMENTAL.

The composition of the basal and other diets is shown in Table I. The casein and the rice supplied about equal amounts of protein, and the level of protein intake was approximately 10 per cent in all the diets. In diets 2 to 5 the calcium content of the basal diet was increased from 0·036 per cent to 0·4 per cent by the addition of calcium lactate, the diets being similar in other respects. According to Sherman and Booher (1931), the percentages of calcium in diets 'used in a colony for numerous families of rats throughout several generations' were 0·19 and 0·33 respectively. These workers reported that increase of calcium intake above these levels (to 0·5 per cent) did not have any effect on the growth rate although it increased the calcium content of the skeleton. It may be assumed that in the present experiments

the lower levels of calcium (0·011, 0·036, and 0·091 per cent) were below the requirements of the rat, while the higher intakes (0·108 and 0·400 per cent) approximated to normal or optimum requirements. The amount of calcium contained in a quantity of the experimental diets yielding 2,600 calories, or approximately the daily requirements of an adult man, is shown in Table I. It will be seen that, if a human being consumed the experimental diets in the proportions indicated, his calcium intake would rise from a point far below his generally accepted requirement—which may be roughly stated as 0·5 g.—to a point considerably above this level.

TABLE I.

Composition of the diets.

Ingredients of the diet.	Diet 1, basal diet, g.	Diet 2, g.	Diet 3, g.	Diet 4, g.	Diet 5, g.	Diet 6, nitrogen-free diet, g.
Rice, raw, milled ..	87·0	87·0	87·0	87·0	87·0	..
Casein (purified) ..	5·0	5·0	5·0	5·0	5·0	..
Starch ..	4·0	3·8	3·45	2·9	1·0	82·0
Coco-nut oil ..	10·0	10·0	10·0	10·0	10·0	10·0
Salt mixture (Ca free) ..	2·0	2·0	2·0	2·0	5·0*	5·0*
Calcium lactate ..	..	0·2	0·55	1·1	..	..
Cod-liver oil ..	3·0	3·0	3·0	3·0	3·0	3·0
Protein, per cent ..	10·35	10·35	10·35	10·35	10·35	..
Calcium, per cent ..	0·011	0·036	0·091	0·168	0·400	0·388
Calcium, per 2,600 calories	0·065	0·213	0·541	1·004	2·433	..

(With 4 c.c. of an aqueous solution of yeast extract corresponding to 1 g. of dried yeast daily to each rat.)

* McCollum's salt mixture containing calcium (5 g. supplied 0·4 g. calcium).

The two well-known methods of determining the biological value of proteins were employed : (1) the nitrogen-balance method for the 'maintenance' of nitrogenous equilibrium using adult rats, and (2) the growth method using young rats. In the latter method, in which the experiment lasted for a period of 4 weeks, the rats were kept in metabolism cages and the nitrogen metabolism also was followed. Full details regarding the technique have already been published (Swaminathan, 1937*a, b*). The data regarding the metabolism and growth experiments are given in Tables II and III respectively. In the balance experiments (Table II) the same 5 animals were used for all experiments, the figures given representing an average for the 5. In the growth experiments (Table III) each rat group consisted of 6 animals and again mean values are given. The results obtained by both methods are summarized in Table IV.

TABLE II.

Nitrogen-balance experiments : biological values and digestibility co-efficients.

(Figures of intake and excretion represent the daily average for the groups on different diets.)

Period number.	Diet number.	Protein in diet.	Calcium in diet.	Phosphorus in diet.	Initial body-weight.	Final body-weight.	Food intake.	Nitrogen intake.	Urinary N.	Faecal N.	Body N saved.	Food N absorbed.	Biological value.	Digestibility co-efficient.
1	6	...	0·388	0·416	138	133	10·82	...	41·3	16·3	...	...	...	...
2	1	10·35	0·011	0·358	139	144	11·17	184·9	99·8	26·9	118·6	175·4	67	95
3	2	10·35	0·036	0·358	146	149	11·21	185·6	104·6	27·8	113·3	174·7	65	94
4	3	10·35	0·091	0·358	154	157	11·04	182·9	106·4	26·6	110·1	173·3	63	95
5	4	10·35	0·168	0·358	157	157	11·51	190·5	107·1	26·5	117·1	181·1	64	95
6	5	10·35	0·400	0·516	158	160	11·58	192·0	105·4	26·4	120·2	182·4	66	95
7	6	...	0·388	0·416	154	143	11·15	...	45·0	17·7	...	...	...	...

TABLE III.

Growth experiments.

(Figures of intake and excretion represent the average of each group for the period of 28 days.)

Rat group number.	Protein in diet, per cent.	Calcium in diet, per cent.	Phosphorus in diet, per cent.	Initial body-weight, g.	Final body-weight, g.	Food intake, g.	N intake, g.	NITROGEN EXCRETION. Urinary, g.	Fæcal, g.	NITROGEN RETENTION. g.	Expressed as percentage of intake.	Biological value (gain per gramme of protein intake).
I ..	10·35	0·011	0·358	60	84	192·7	3·191	1·574	0·435	1·182	37	1·20
II ..	10·35	0·036	0·358	60	96	192·3	3·184	1·348	0·445	1·391	44	1·81
III ..	10·35	0·091	0·358	58	94	201·4	3·335	1·600	0·463	1·272	38	1·73
IV ..	10·35	0·168	0·358	59	94	194·3	3·218	1·625	0·425	1·168	36	1·74
V ..	10·35	0·400	0·516	59	101	212·0	3·511	1·520	0·523	1·468	42	1·91

TABLE IV.

The average weekly increase in body-weight and the biological value of proteins.

Diet number.	Protein in diet, per cent.	Calcium in diet, per cent.	Average weekly increase in body-weight, g.	Biological value (by the growth method).	Biological value (by the nitrogen-balance method).
1	10·35	0·011	6·0	1·20	67
2	10·35	0·036	9·0	1·81	65
3	10·35	0·091	9·0	1·73	63
4	10·35	0·168	8·8	1·74	64
5	10·35	0·400	10·5	1·91	66

DISCUSSION.

The level of calcium intake did not appear to have any influence on the values obtained by the nitrogen-balance method with adult rats. While it is possible that a very low or high calcium intake over a long experimental period might influence the nitrogen balance, the usual duration of experiments of this nature is probably too short for any such effect to be produced. With regard to the growth experiments, it was found that growth was retarded when the calcium intake was extremely low (0·01). A level of calcium as low as 0·036 per cent in diets containing 10 per cent of protein and adequate in other respects, allows approximately normal growth in young rats during a period of 4 weeks. Further increase in the calcium content of the diet up to 0·4 per cent does not appreciably affect the increase in body-weight per gramme of protein ingested.

Toepfer and Sherman (*loc. cit.*) determined the gain per gramme of protein in a series of rat growth experiments in which intake of calcium was varied. The gain per gramme of protein was uninfluenced by the percentage of calcium in the diet, so that their findings are in agreement with those reported in this paper. Their results are summarized in Table V.

TABLE V.

Gain in weight per gramme of protein at varying levels of calcium intake
(Toepfer and Sherman, loc. cit.*).*

Diet number.	Protein in diet, per cent.	Calcium in diet, per cent.	Phosphorus in diet, per cent.	GAIN IN BODY-WEIGHT.		Gain per 1,000 calories, g.	Gain per gramme of protein, g.
				Males, g.	Females, g.		
16	13·5	0·2	0·4	65 ± 2*	53 ± 2	71·8 ± 0·5	1·99 ± 0·06
168	13·4	0·64	0·4	76 ± 2	75 ± 2	74·5 ± 0·9	2·05 ± 0·03
169	13·3	0·80	0·53	70 ± 2	62 ± 1	77·6 ± 0·6	2·14 ± 0·01
268	13·3	0·80	0·4	64 ± 2	57 ± 1	66·3 ± 0·6	1·86 ± 0·02

*Probable error.

SUMMARY.

The biological values of a mixture of casein and rice proteins, as determined by the standard nitrogen-balance and growth methods, were not affected by changes in the calcium content of a series of experimental diets. The calcium content of the diets ranged from 0·036 to 0·4 per cent.

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SERUM-PHOSPHATASE ACTIVITY IN RATS FED ON A POOR SOUTH INDIAN DIET VARIOUSLY SUPPLEMENTED.

BY

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THE superiority of serum-phosphatase estimation over phosphorus and calcium determination for the diagnosis of rickets is now well recognized. Recent work has revealed certain relationships between the activity of blood phosphatase and certain dietary deficiencies. Typical poor South Indian diets are very deficient in mineral salts, particularly calcium ; in a series of experiments on rats it was found that the addition of calcium lactate to such diets enhances growth and improves the general condition of the rats (Aykroyd and Krishnan, 1937). It was, therefore, thought that a study of the changes in serum-phosphatase activity which might occur when such diets are consumed would be of interest. The present paper describes an investigation of the phosphatase activity of the blood serum of rats fed on a poor South Indian diet supplemented by calcium lactate and excess of fat.

EXPERIMENTAL.

Blood was drawn from the abdominal aorta of the rats under ether anæsthesia, the animal being subsequently killed. The serum was prepared immediately by centrifuging.

The phosphatase activity was determined according to the method described by Bodansky (1937) with slight modifications. In the method described by Bodansky, magnesium salt is not used for activation of the phosphatase. Since magnesium is an essential activator for the alkaline phosphatases of tissues and blood, the influence of magnesium on serum phosphatase of rats, and the optimum concentration of magnesium necessary for the activity of the phosphatase, were determined. 0.01 mol. $MgCl_2 6H_2O$ was found to be the optimum concentration for the activity of the phosphatase, and in all the determinations of activity the magnesium concentration in the reaction mixture was adjusted to this value.

The buffered substrates were made up daily in the proportions described below :—

    45   c.c. of N/10 glycine-NaCl mixture
     5   c.c. N/10 NaOH
    0·5 gr. sodium-β-glycerophosphate (Merck)
    50   c.c. water.
            Total volume = 100 c.c.   pH = 8·9.

DETERMINATION OF PHOSPHATASE ACTIVITY.

Ten c.c. of the buffered substrate mixture were taken in a series of test-tubes and 1 c.c. of magnesium chloride solution and 0·5 c.c. of water were added to each. The test-tubes were tightly stoppered and kept in a thermostat at 35°± 0·1°C. After the mixture attained the temperature of the bath, 0·5 c.c. of serum was added, and the digestion was allowed to proceed for 1 hour.   At the end of this period 8 c.c. of 10 per cent trichloracetic acid were added and the contents filtered through No. 44 Whatman filter-paper.   The inorganic P in the filtrate was estimated by the method of Fiske and Subbarow (1925).   The inorganic P originally present in the serum was also determined.   The difference between the two values represents the activity of the phosphatase.   Bodansky's table and factors to allow for the effect of Beer's law and for the influence of β-glycerophosphate and trichloracetic acid on the colorimetric readings were used for the calculation of the activity in Bodansky's units.

ACTIVATION OF SERUM-PHOSPHATASE ACTIVITY BY MAGNESIUM.

In the method described by Bodansky for the determination of the phosphatase activity of human serum, magnesium salt was not added to the digestion mixture. Since magnesium is an indispensable activator for the phosphatases of animal tissues in general, it was thought that the addition of magnesium salt in optimal amounts to the reaction mixture would be necessary for obtaining a correct value of the activity.   In Table I are presented the results of experiments on the influence of magnesium as $MgCl_2 6H_2O$ on the serum-phosphatase activity of rats :—

TABLE I.

Influence of magnesium on serum-phosphatase activity.

Magnesium concentration.	Phosphatase units.
0	14·2
0·0005 M ..	16·8
0·005 M ..	21·5
0·010 M ..	21·6
0·020 M ..	20·8

The results recorded in the above table show that the optimum concentration of magnesium salt for complete activation lies at 0·005 mol. to 0·01 mol. In subsequent experiments, the activity was determined in the presence of 0·01 mol. concentration of magnesium chloride.

Changes in serum-phosphatase activity in preserved serum.—In Table II are presented the results of observations on the effect of preserving serum at 0°C. on the activity of the phosphatase both in the presence and in the absence of magnesium.

TABLE II.

Serum-phosphatase activity in preserved serum.

	SERUM-PHOSPHATASE UNITS.	
	Without magnesium.	With magnesium (0·01 mol.).
1. Fresh serum No. I	14·2	21·6
Preserved serum No. I (kept at 0°C. for 24 hours).	17·8	23·4
2. Fresh serum No. II	19·2	30·2
Preserved serum No. II (kept at 0°C. for 24 hours).	25·0	30·0

The results show that the activity in the absence of magnesium is increased, while the activity in the presence of magnesium is not much influenced on keeping the serum at 0°C. for 24 hours. Bodansky (1937) also observed 7 to 23 per cent increase in phosphatase activity when blood serum was kept at room temperature for 1 hour and 23 hours in the refrigerator. It is clear, therefore, that the phosphatase of serum on keeping at 0°C. appears to respond less readily to magnesium activation than the phosphatase of fresh serum. These findings are in conformity with the previous observations made by Giri (1938) who showed that the phosphatase of liver, kidney, and brain are less susceptible to magnesium activation when the aqueous extracts are kept for some time at room temperature.

The serum-phosphatase activity of rats fed on the poor South Indian diet and the same diet supplemented by calcium lactate and fat.—Five groups of rats, weighing about 50 grammes, were fed on the following diets :—

GROUP   I : Poor South Indian diet.*
,,   II :   ,,   ,,   ,,   ,,   + calcium as calcium lactate (0·08 per cent Ca supplement).
,,   III :   ,,   ,,   ·,   ,,   + calcium lactate (0·16 per cent Ca supplement).
·,   IV :   ,,   ·.   ,,   ,,   + gingelly oil (10 per cent).
,,   V :   ,,   ,,   ,,   ,,   + lard (10 per cent).

The calcium supplements were roughly equivalent to 0·01 gramme and 0·02 gramme per rat per day, respectively.

Water and food were given *ad libitum.* The serum-phosphatase activity was determined after eight weeks. In Table III the results of these experiments are presented :—

TABLE III.

Serum-phosphatase activity of rats fed on the poor South Indian diet, supplemented with calcium lactate and fat.

Diet.	Rat number.	SERUM-PHOSPHATASE UNITS.	
		Without magnesium.	With magnesium.
Poor South Indian diet	1—2	28·4	37·5
	3—4	29·0	43·8
Poor South Indian diet + 0·08 per cent Ca as calcium lactate.	5—6	9·2	10·9
	7—8	17·2	17·2

* The composition of the diet is as follows (Aykroyd and Krishnan, *loc. cit.*) :—

Grammes.

Raw polished rice	600·0
Dhal arhar (*Cajanus indicus*)	20·0
Black gram (*Phaseolus mungo*)	20·0
Gingelly oil	2·8
Brinjal (*Solanum melongena*)	28·0
Amaranth (*Amaranthus gangeticus*)	14·0
Raw plantain (*Musa paradisiaca*)	14·0
Mutton	1·7
Coco-nut (*Cocos nusifera*)	1·4

TABLE III—*concld.*

Diet.	Rat number.	SERUM-PHOSPHATASE UNITS.	
		Without magnesium.	With magnesium.
Poor South Indian diet + 0·160 per cent Ca as calcium lactate.	9—10	19·0	26·4
	11—12	18·4	26·8
Poor South Indian diet + gingelly oil (10 per cent).	13—14	64·2	85·2
	15—16	88·9	117·2
Poor South Indian diet + lard (10 per cent) ..	17—18	46·0	59·6

It can be seen from Table III that the addition of calcium lactate to the basal diet lowers the serum-phosphatase activity, while the addition of fat has the opposite effect.

With a view to confirming the effect of fat on serum-phosphatase activity, another experiment was carried out using young rats weighing about 50 grammes. The rats were divided into three groups. The first group was given the basal diet, while the second and third groups received the same diet supplemented with varying amounts of fat. Serum-phosphatase activity was determined after three weeks. The results are presented in Table IV :—

TABLE IV.

Influence of additional fat on serum-phosphatase activity.

Diet.	Total fat in the diet. Per cent.	Serum-phosphatase units (in the presence of 0·01 mol. Mg.). Average for 3 rats.
Poor South Indian diet	0·4	22·0
Poor South Indian diet + gingelly oil ..	2·0	43·1
Poor South Indian diet + excess of gingelly oil ..	5·0	179·0 (average for six rats).

DISCUSSION.

It is a well-established fact that serum-phosphatase activity is related to bone diseases such as rickets, osteomalacia, osteitis deformans. Recent studies by several workers have shown that the blood phosphatase of young animals is very sensitive to dietary influences. Smith (1933) has shown that the activity of plasma

phosphatase of infants fed on 'artificial diets' was higher than that of breast-fed children. The experiments of Bodansky and Jaffe (1934) on rats have shown that the plasma-phosphatase activity of young growing rats on a Sherman diet was lower than that of rats on a meat diet. Bodansky and Jaffe from their clinical study of children have shown that serum calcium and inorganic phosphorus are not reliable criteria of the severity of rickets or of the rate of healing of rickets, but that the phosphatase values may be used as a criterion of the effectiveness of the treatment. This important relationship between serum-phosphatase activity and bone diseases has led many workers to study the effect of diets which are not optimal in Ca and P content on the level of serum phosphatase. Actually rickets is not very common in South India. The poor South Indian diet is however in general deficient in the important mineral calcium and the effect of such a diet is to produce a high serum-phosphatase activity.

Auchinachie and Emslie (1933) have shown that disordered metabolism of Ca and P is associated with a marked increase of plasma phosphatase in sheep. This increase, which occurred in animals on an inadequate diet, took place appreciably earlier than the fall in serum Ca, and before any other sign of disordered metabolism had appeared. Thus, the change produced by a deficient diet in the plasma phosphatase in sheep provides a much earlier indication of disordered Ca and P metabolism than either the serum Ca, the blood inorganic P, the general state of health, or the body-weight. Diets deficient in Ca were found to produce an increase in the plasma phosphatase in these animals. Poultry (1934) also found that the plasma phosphatase of sheep on diets low in Ca but high in P increased considerably. The results obtained in the present study on the influence of calcium lactate on serum-phosphatase activity of rats fed on the poor South Indian diet are in conformity with the findings of the above workers. Another interesting finding is the considerable increase in the serum-phosphatase activity of rats fed on excess of fat. The physiological significance of this is not yet clear.

SUMMARY.

The serum-phosphatase activity of rats fed on a poor South Indian diet was higher than that of rats receiving the same ration supplemented with calcium lactate. Addition of extra fat to the basal diet was found to increase the serum-phosphatase activity.

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A CONTRIBUTION TO THE STUDY OF THE PATHOGENESIS OF PEPTIC ULCER IN INDIANS.

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PEPTIC ULCER in India has a peculiar geographical distribution. While gastric and duodenal ulcers are very common in South India, they are rare in North India. From a study of the hospital returns from different parts of the country, McCarrison (1936) has shown that peptic ulcer (gastric and duodenal) is 58 times more common in the South than in the North. Somervell and Orr (1936) have recorded its frequency in Travancore State in South India. Bradfield (1927) made some interesting observations on the subject. He found that ulcers of the stomach and duodenum are very common in South India and occur amongst every race and caste.

The cause of the greater incidence of the disease in South India is at present unknown. Several factors may be responsible. One of the important differences between the races of the North and South is in dietary habits. In general, the diet of the people of large areas in North India consists of 'chapatties' made of whole-wheat flour, and contains milk and milk products, and pulses in fair abundance. In South India rice is the staple article of diet; milk and milk products, vegetables, and fruits enter into the diet but sparingly. In Travancore, a considerable proportion of the population consumes tapioca in combination with rice. It may thus be said that in general the diet of the wheat-eating peoples of the North is richer in protein and in certain vitamins and minerals than that consumed in the South.

While there is a general impression that dietary defects may either pre-dispose to or be in some way causally related to peptic ulcer, no convincing evidence has been brought forward to substantiate this view.   As a preliminary approach to the problem, the pathological changes occurring in the stomach and duodenum of individuals suffering from peptic ulcer and normal individuals have been studied in detail. Similar investigations have been carried out on animals fed on various types of deficient diet.

MATERIAL AND METHOD OF INVESTIGATION.

The clinical material for investigation was obtained from various Government and Mission Hospitals in South India.   Much of this was obtained during laparotomy for peptic ulcer. Through the co-operation of Civil Surgeons in Travancore, Malabar, and North India, autopsy material for study was collected.   The clinical and autopsy material may be divided into the following groups :—

A. *Clinical material.*—(*i*) During operations for the re-section of duodenal ulcer, a small piece of the stomach, at a distance from the ulcer, was removed in order to discover whether any variations from the normal were present in the mucosa and muscle of the stomach as a whole.

(*ii*) A portion of the stomach or duodenum proximal or distal to the pylorus was excised for histological investigation from patients subjected to laparotomy for suspected ulcer, but in whom no ulcer was found on operation.

B. *Post-mortem material.*—In order to provide a standard of comparison for changes found in the stomach at a distance from ulcers, an effort was made to collect post-mortem material from persons in South India dying a violent death. e.g., as a result of accident, hanging, or post-operative shock. Similar autopsy material was also collected in North India.

C. *Experiments with rats.*—Groups of young albino rats obtained from the healthy and well-fed stock of the Coonoor Laboratories were fed on the following diets :—

(*i*) *A diet based largely on raw milled rice resembling in composition that consumed by human beings in certain parts of South India.*—The composition of the diet, which is known in the Laboratories as the ' cheap Madrassi diet ', was as follows :—

	Oz.	G.
Raw milled rice	21·00	596
Dhal arhar (*Cajanus indicus*)	0·70	20
Black gram (*Phaseolus mungo*)	0·70	20
Gingelly oil (*Sesamum indicum*)	0·10	3
Brinjal (*Solanum melongena*)	1·00	28
Amaranth (*Amaranthus gangeticus*)	0·50	14
Raw plantain (*Musa paradisiaca*)	0·50	14
Meat (*Mutton*)	0·06	1·7
Coco-nut	0·05	1·4

(ii) *A diet consisting mainly of tapioca and rice, with various additions.*—
This diet in general *resembles* that consumed in the tapioca-producing areas
of South India.

	Oz.	G.
Tapioca root (*Manihot utilissima*)	10·000	284
Parboiled rice	10·000	284
Chillies (*Capsicum annuum*)	0·125	3·5
Tamarind (*Tamarindus indicus*)	0·125	3·5
Raw plantain	0·500	14
Brinjal	0·500	14
Coco-nut oil	0·250	7

The animals were killed after different periods of feeding on the above
diets and pathological investigation of the stomach and duodenum carried out.
The period of feeding before the animals were killed and examined varied from
8 to 18 months. Albino rats of roughly the same age from the stock served as
controls. The latter received a good ration consisting of ' atta (whole wheat)
chapatties ' smeared with butter, fresh raw cabbage, fresh raw carrots, sprouted
Bengal gram, cow's fresh raw milk, and meat, twice a week.

D. *Experiments with dogs.*—Seven dogs were kept in moderate confinement
with regulated exercise and given a fairly well-balanced diet based on rice, meat,
bread *ad lib.*, and milk two ounces daily. These experiments were carried
out in Miraj (S. India). Each dog was anæsthetized and, under suitable aseptic
conditions, portions of the duodenum and pyloric end of the stomach were
removed to study the normal histology of these organs, and the gap closed by
suturing. After a suitable interval to permit of recovery from the operation,
the dogs were fed on an extremely deficient diet consisting mainly of tapioca.
At the end of two and four months' feeding on this diet, the animals were
subjected to laparotomy and biopsy as before. Pieces of stomach and duodenum
were removed for pathological examination, the scars left by the previous
sections being avoided.

The tissues so obtained were fixed in formol saline. Preparations for histo-
logical study were made in the usual manner, employing paraffin embedding and
staining by Ehrlich's acid hæmatoxylin and eosin. The rats were killed by air
embolism and the organs removed and fixed immediately after death. The
biopsy material from dogs and most of the human material was similarly fixed
immediately after removal. During the later part of the investigation, prepara-
tions were made both from the animal and human specimens using a modified
Nissl staining technique, in order to study the cytological changes in the
ganglion cells of Auerbach's plexus.

DESCRIPTION OF FINDINGS.

A. (i) *Sections obtained by biopsy from cases of peptic ulcer.*—Of the 39 stomachs
re-sected, only four showed a normal mucosa. The mucous membrane in the re-
maining 35 specimens showed varying degrees of infiltration with plasma cells and
lymphocytes. In the more advanced degrees of invasion, the normal mucosal
glands were widely separated and reduced in number, the intervening spaces being

crammed with round cells. In the deeper part of the mucosa, aggregation of lymphoid elements occurred to form follicles and, in some instances, the whole of the sub-mucosal space was one lymphoid area with aggregation into follicles at intervals.

The lymph follicles frequently showed abscess formation with spaces in the centre filled with necrotic cells. In some sections the follicles were seen to have ruptured and a cleft or erosion in the mucosa extended down to the crater of the follicle.

Round-celled infiltration of the muscle layers was present in some cases, but never to a severe degree.

Changes in Auerbach's plexus.—The following description of the normal Auerbach's plexus is based on our own observations and data contained in various textbooks. The plexus is found in the fibrous septum between the longitudinal and circular layers of the muscular coat of the gastro-intestinal tract and consists of numerous small ganglia, united by small bundles of nerve fibres, most of which are non-medullated. The nerve cells of the plexus vary much in size and shape and are grouped to form the ganglia. These ganglia are not easy to find in normal tissue. Each ganglion consists of three or four, or sometimes more, nerve cells surrounded by some endothelial cells or glial cells (Holsti, 1931). The nerve cells are larger than any other cells in the preparation; they are pear-shaped or oblong with one end of the oblong rounded. They contain a large, clear, spherical nucleus with well-formed nucleoli, usually solitary, and a 'pattern' of chromatin rods and dots. The nucleus is refractile and is generally centrally placed and surrounded by a well-marked membrane.

In sections stained by Nissl's method, finely dispersed Nissl granules are seen in the cell cytoplasm.

Some of the tissues received during the early part of the investigation could not be used for a study of the plexus, as these were not fixed and prepared in a manner suitable for the demonstration of changes in ganglion cells. Changes in the ganglion cells occur very early after death and only tissues which have been fixed immediately post mortem provide reliable material for study. For satisfactory examination, it is necessary to have tissues taken at operation or from animals killed by air embolism, and fixed immediately.

In 25 suitable specimens studied, the following points were noted :—

(1) In no case was a normal plexus observed, though isolated groups of normal cells were seen in several specimens.

(2) In most cases the plexus was enlarged, œdematous, and infiltrated with round cells and fibroblasts. The normal glial cells of the plexus were increased. Cellular infiltration of the plexus was, however, not a constant feature and was absent in some cases showing considerable degeneration of the ganglion cells. A thick capsule was sometimes observed around the degenerated plexus.

The ganglia of Auerbach's plexus were easily recognized in the sections, due to the general swelling of the plexus or atrophy of the ganglion cells. In the normal section recognition was more difficult.

Varying degrees of degenerative changes were found in the ganglion cells. Such differences in degree were often present in the same specimen or sometimes in the same ganglion. The changes varied from cloudy swelling (chromatolysis) to complete degeneration of the cell.

The cells were often swollen, the cell outlines indistinct or uneven, and the cytoplasm homogeneous. In sections stained by Nissl's method, the cytoplasm was stained an even blue colour devoid of granules. The nuclei were swollen and did not stain well ; in some instances, they were distorted and showed a defective nuclear membrane. In some cells the nuclei were shrunken, eccentric in position, and granular in appearance ; the nucleoli were indistinct with the Nissl granules collected round the nucleus. In others the nucleoli were fragmented or absent.

A more advanced degree of degeneration was present in some cells. The cells were smaller than normal, vacuolated, and shrunken ; the nuclei were pyknotic, pushed to one side of the cell or partly extruded, and appeared elongated and flattened. Some cells showed complete disintegration of chromatin and disappearance of the nucleus and only skeletons of dead or degenerated cells were observed. In the more advanced cases, the ganglion cells had completely disappeared, leaving empty spaces in the ganglion which appeared as a large syncytial mass infiltrated with round cells and fibroblasts.

The number of ganglion cells involved in the degenerative process and their distribution in the plexus varied considerably in the several specimens. In some ganglia, normal cells were seen alongside degenerated cells, showing that the changes observed were not due to defects in histological technique. Groups of ganglion cells have disappeared in some specimens, while in some advanced cases marked changes were found in a large number of cells of the plexus.

(*ii*) *Biopsy material from cases with symptoms of ulcer, but no demonstrable ulcer at operation.*—One of us (I. M. O.) has encountered from time to time cases which give a history very like of peptic ulcer, in which no ulcer is demonstrated by X-ray examination. The duodenal cap, however, is found to fill with difficulty as a result of pyloro-spasm preventing the stomach contents from passing freely into the duodenum. When the duodenal cap does fill, it presents an irregular outline and empties rapidly and is tender on pressure. This condition is labelled as ' irritable duodenum ' by the radiologist and is frequently associated with powerful and rapid peristalsis of the antrum. It has been described by Garry (1937) and Friedenwald and Feldman (1934).

In spite of an indefinite X-ray report, the symptoms and signs simulated ulcer so closely in 10 patients that finally a laparotomy was undertaken. No ulcer was found but a small portion of the duodenum or the antrum proximal to the pylorus was removed for histological study.

In all 10 specimens, the mucosa, particularly the superficial part, was infiltrated with lymphocytes. In six cases, aggregations of lymphoid elements to form follicles were found and some of the follicles showed evidence of bursting on to the surface of the mucosa. Occasionally, an erosion of the surface mucosa led down to an erupted follicle.

Changes in Auerbach's plexus identical with those described in the previous section were also found in this group. In only one specimen in this series were there found normal ganglion cells in large numbers; even in this specimen, groups of cells in the earlier stages of degeneration were found and the invasion by round cells and swelling of the ganglia betrayed commencing degenerative changes.

B. Post-mortem material from persons in South and North India with no history of ulcer.—Fresh post-mortem material is exceedingly difficult to obtain in India owing to the religious and social customs of the people. Eight good specimens (stomach and duodenum) were obtained, however, in South India, the subjects being criminals after execution or persons dying in hospitals from the effects of violence or post-operative shock.

As none of the above material was especially collected and preserved with a view to showing the nerve plexus in good condition, changes in the ganglion cells could not be studied in six cases as the possibility of post-mortem degeneration could not be excluded. In two cases, however, in which a post-mortem examination was carried out very soon after death, specimens suitable for this purpose were obtained.

As in the previous groups, all the sections showed round-celled infiltration of the mucosa and one showed hyperplasia of lymphoid follicles similar to that seen in the definitely pathological cases.

The two specimens in which Auerbach's plexus was investigated are of special interest. Both were taken from the stomachs of persons who died under an anæsthetic from an operation for a condition in no way related to the gastro-intestinal tract. Both showed early degeneration of the ganglion cells of Auerbach's plexus. Only one showed any marked degree of lymphocytic infiltration of the mucosa.

Seven specimens were collected from post-mortem examinations made shortly after death in North India. These showed a normal healthy mucosa. No lymphoid follicles were observed and no evidences of inflammation were present in the mucous membrane. In two specimens in which Auerbach's plexus was demonstrated, it appeared to be normal.

C. The stomach and duodenum of rats fed on the ' cheap Madrassi diet' and the ' tapioca diet'.—The rats were weighed at weekly intervals. The animals failed to grow satisfactorily and lost weight in the later part of the experiment. Those that had any coincident disease were rejected as not being suitable for a study of the nerve cells.

The following observations were made :—

1. None of the animals showed ulcer of the stomach or duodenum.

2. Inflammatory changes in the mucosa of the stomach or duodenum were slight or absent in the rats fed on the deficient diets for less than six months. Mild inflammatory changes such as round-celled infiltration of the mucosa were found occasionally in the animals fed on the diets for a year or more. Aggregation of the lymphoid cells to form follicles were present only in rare instances.

3. Degenerative changes in Auerbach's plexus were progressive and could be definitely demonstrated even in rats fed on the deficient diets for a few months only. In general, rats, fed on the 'tapioca diet', showed the changes earlier than the animals fed on the 'cheap Madrassi diet'. In both groups the degenerative changes in the plexus were roughly proportional to the period of deficient feeding. These changes are similar to those found in human cases of ulcer. Hyperplasia of glial cells and infiltration of the ganglia by round cells and fibroblasts were, however, rarely present.

In well-fed stock animals the nerve cells of the plexus appeared in groups of three to five cells, with large clear nuclei and well-marked nucleoli and nuclear membrane. Pyknosis of the nucleus or other evidences of degeneration were, however, occasionally found. The cell bodies could be demonstrated in most instances even by the ordinary staining methods. Few glial cells were present in the ganglia, but no fibroblasts were seen. On the whole the differences in the appearance of the plexus in the well-fed and deficiently-fed groups were striking.

D. Experimental material from dogs fed on a fairly well-balanced diet followed by a 'tapioca diet'.—In the specimens of the stomach and duodenum removed before the tapioca diet was given, a healthy mucosa was seen. In one case, however, slight round-cell infiltration of the gastric mucosa, suggestive of early gastritis, was present. Auerbach's plexus appeared normal and closely resembled the plexus in the normal human stomach and duodenum. The ganglion cells were accompanied by few glial cells and there was little or no infiltration by lymphocytes, plasma cells, or fibroblasts. In the normal plexus, an occasional pyknotic or degenerative form was seen but the great majority of the cells conformed to the normal as described in the preceding sections.

All the dogs fed on the tapioca diet lost weight and appetite. Two animals developed ascites and two finally died of intussusception. At the end of four months of deficient feeding, the animals which survived were emaciated and ill and hence allowed to die under the anæsthesia after biopsy.

None of the dogs developed ulcer of the stomach or duodenum.

Examination of the specimens removed at the end of two and four months respectively showed definite changes in the plexus. The ganglion cells were pyknotic or degenerative; there was a great increase in the fibrous tissue and the plexus was surrounded by a capsule. Marked infiltration of the ganglia by round cells was observed and the condition of the plexus became similar to that found in human cases of ulcer.

While the plexus changes were definite and constant, the changes in the mucosa were varied in degree. A tendency to increased round-celled infiltration and hyperplasia of lymph follicles was noted but the marked gastritis and duodenitis of the human ulcer cases were not observed.

The changes in the plexus and the mucosa lesions were not parallel as regards time of appearance and intensity. The former, in general, appeared earlier; in some specimens, which showed marked plexus degeneration, no noteworthy changes in the mucosa could be discovered. In one case, already referred to, the first or

'healthy' section showed early gastritis but the ganglion cells were normal. The plexus changes are thus not the direct result of a gastritis or duodenitis, but may precede such conditions.

Plates II to VII illustrate the changes described in the preceding sections. Figs. 1 to 14 show changes in the mucous membrane and plexus in human cases. Figs. 17, and 21 to 23 illustrate the normal appearance of the plexus in dogs and rats respectively.

DISCUSSION.

Definite pathological changes were found in the mucosa and the intra-mural nerve plexus of the stomach and duodenum at a considerable distance from the peptic ulcer. The lesions of the mucosa—round-celled infiltration and an increase in the size and frequency of lymphoid follicles—are characteristic of chronic gastritis or duodenitis and may be either the precursor, the accompaniment, or the result of ulcer formation. The presence of similar changes in specimens from individuals complaining of symptoms suggestive of ulcer, but in whom no ulcer crater could be demonstrated by X-rays or at operation, suggests that the chronic inflammatory condition precedes ulcer. This view is supported by the fact that many persons who suffer from typical ulcer symptoms and are operated on without any ulcer being found, undergo a second operation a year or more later, a typical ulcer having developed in the interval.

It is, however, important to note that similar appearances were observed in post-mortem material obtained from individuals not complaining of symptoms referable to gastro-duodenal lesions. This suggests that such changes occur widely in the general population and that *per se* they do not necessarily give rise to peptic ulcer.

Duodenitis and gastritis, with similar histological appearances to those observed by us in the human cases, have been described by several workers (Faber, 1927, 1935; Johnston, 1934; Friedenwald and Feldman, *loc. cit.*; Fitzgerald, 1931; Wellbrock, 1930; Judd and Nagel, 1927; Kellog, 1933; Konjetzny, 1923; and others). Smith (1902-3) records that he found on many occasions, when ulcer was suspected, no ulcer but only small erosions. Microscopically, these were follicular abscesses which ruptured and were thought to be the beginning of ulcers. Simonds (1938) described the mode of origin of experimental gastric ulcer induced by cinchophen. The following sequence is interesting when compared with our findings :—

1. Œdema of single or multiple villi.
2. Diffuse infiltration of villi with plasma cells and lymphocytes.
3. Superficial erosions.
4. Focal accumulation of polymorphs in the villi, just above the muscularis mucosæ often accompanied by liquefactive necrosis.
5. Narrow fistula-like channels extending from such foci to the surface.
6. Large deep ulcers.

PLATE II.
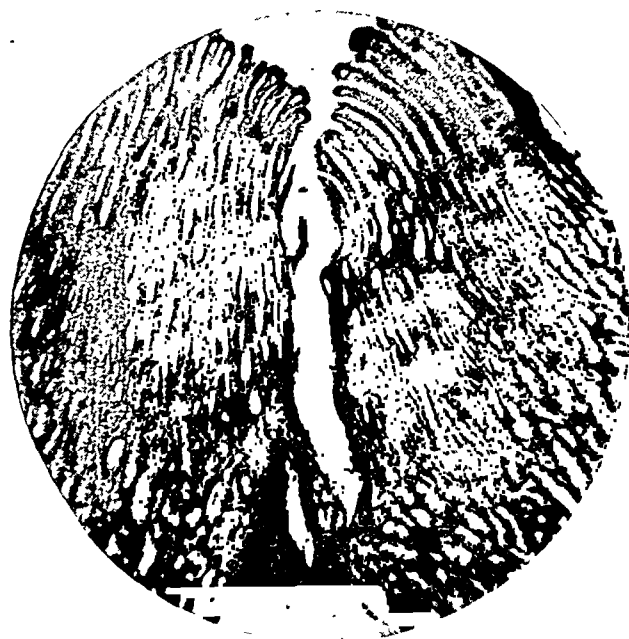
FIG. 1.

Fig. 1. Gastric mucosa from a case of duodenal ulcer showing hypertrophy, lymphocytic infiltration, a follicular abscess. and superficial erosion. × 50.
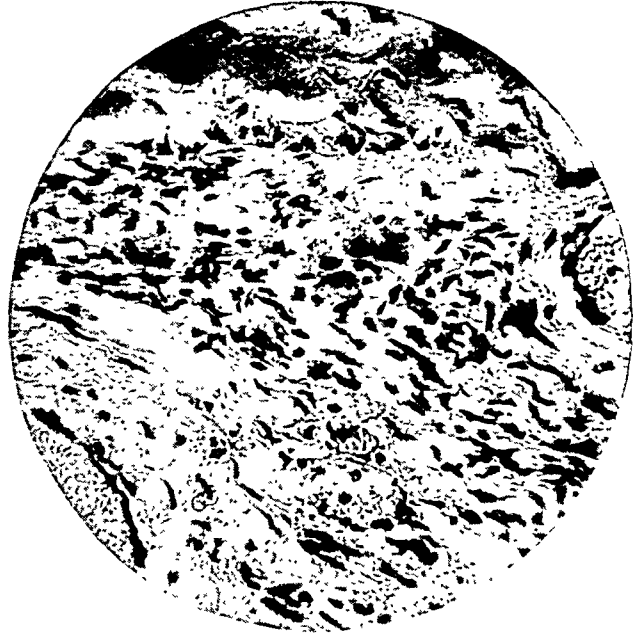
FIG. 2.

Fig. 2. Auerbach's plexus of stomach from a case of duodenal ulcer showing infiltration with round cells and fibroblasts. The nuclei of the ganglion cells are pyknotic, flattened, and pushed to one side of the cell. × 280.

FIG. 3.
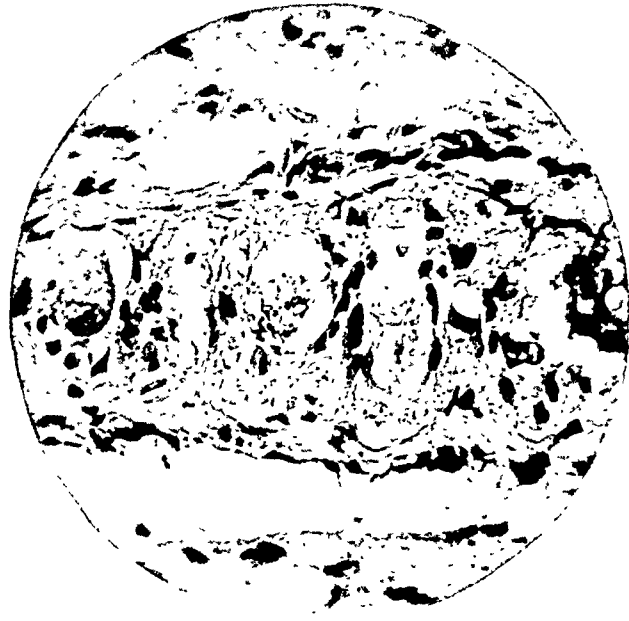
FIG. 4.

Figs. 3 and 4. Sections of stomach from a case of duodenal ulcer showing marked lymphocytic infiltration of the mucosa and a lymph follicle in the deeper part of the mucosa (Fig. 3) and the ganglion cells of Auerbach's plexus show degenerative changes (Fig. 4). Fig. 3. × 50; Fig. 4. × 280.

PLATE III.

FIG. 5.

FIG. 6.

Figs. 5 and 6. Sections of stomach from a case of duodenal ulcer showing a lymph follicle which has very nearly reached the surface of the mucosa (Fig. 5) and cellular infiltration of Auerbach's plexus and degeneration of ganglion cells (Fig. 6). Fig. 5. × 50; Fig. 6. × 280.

FIG. 7.

FIG. 8.

Figs. 7 and 8. Auerbach's plexus of stomach from cases of duodenal ulcer showing cellular infiltration (Fig. 7), fibrosis (Fig. 8), and degenerative changes in most of the ganglion cells (Figs. 7 and 8). Figs. 7 and 8. × 280.

PLATE IV.
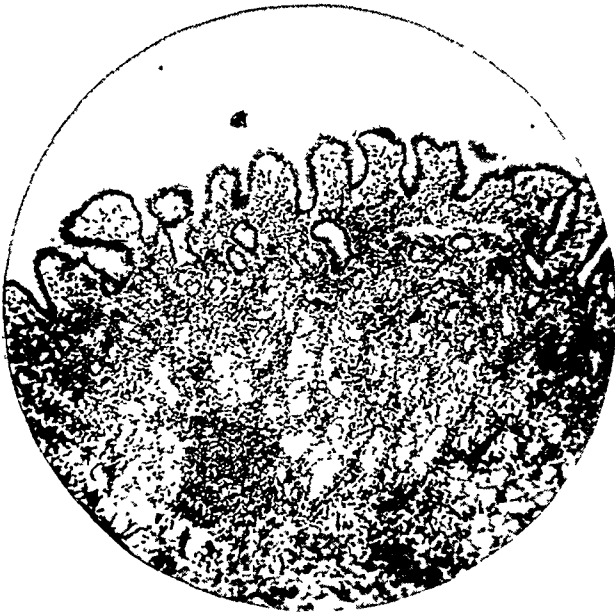
FIG. 9.

FIG. 10.

Figs. 9 and 10. Sections of pyloric antrum from a case with symptoms of ulcer, but no demonstrable ulcer at operation, showing infiltration of the mucosa with lymphocytes and hyperplasia of lymph follicles (Fig. 9); Auerbach's plexus shows degenerative changes (Fig. 10). Fig. 9. × 50; Fig. 10. × 280.
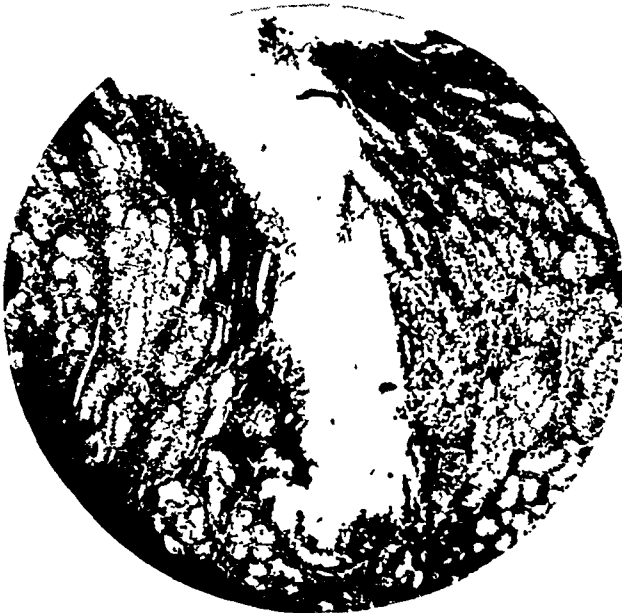
FIG. 11.
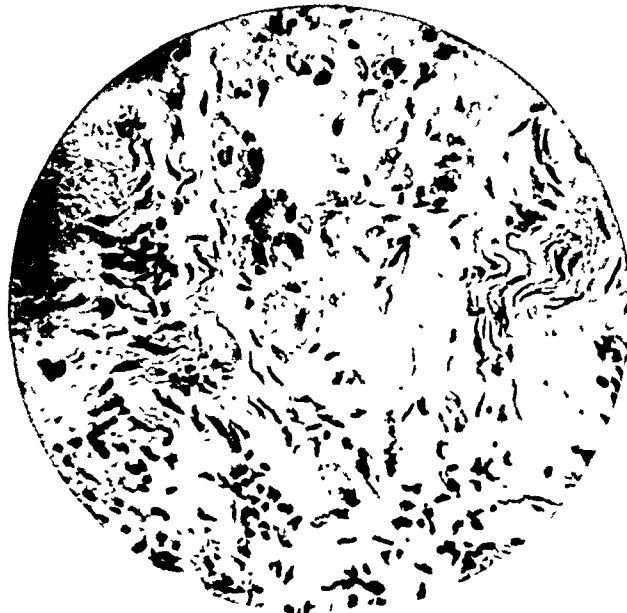
FIG. 12.

Figs. 11 and 12. Sections of duodenum from a person in South India (obtained post mortem), with no history of ulcer, showing round cell infiltration of the mucosa and a lymph follicle which has erupted on the surface forming superficial mucosal erosion (Fig. 11); Auerbach's plexus is swollen and the ganglion cells show degenerative changes. Fig. 11. × 50; Fig. 12. × 280.
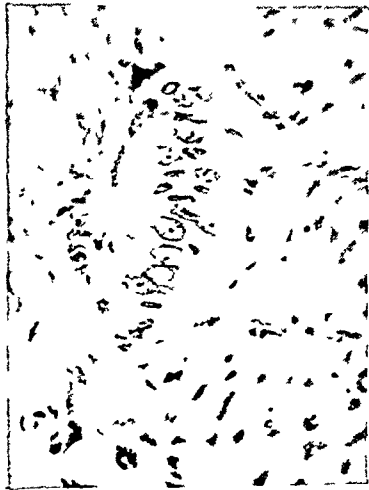
PLATE VII.

FIG. 23.

FIG. 22.

FIG. 21.
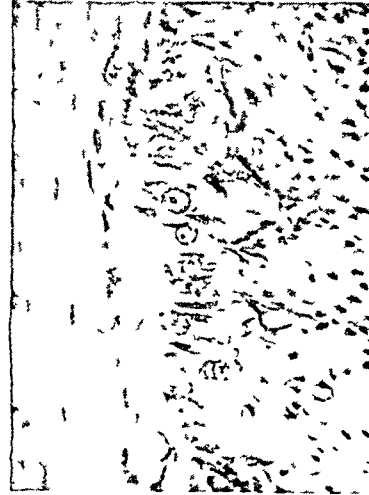
FIG. 26.

FIG. 25.

FIG. 24.
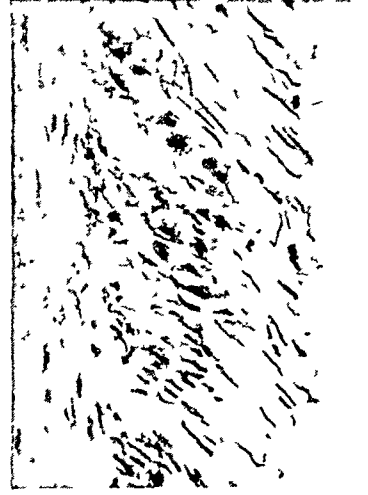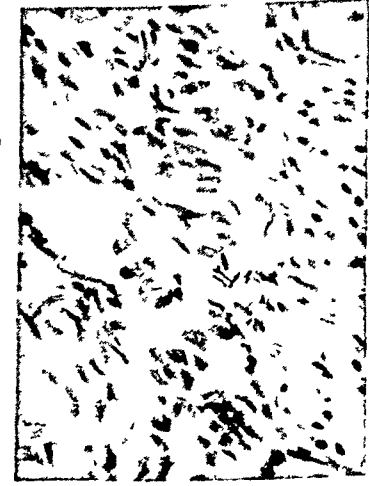
FIG. 27.
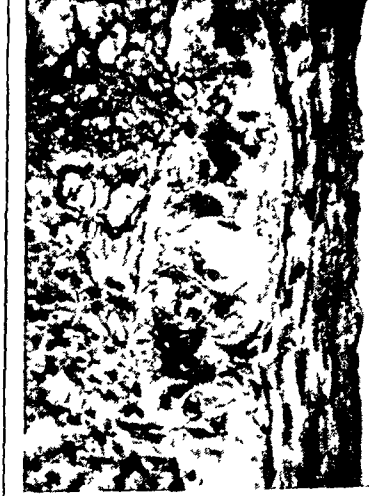
EXPLANATION OF PLATE VII.

Figs. 21, 22, and 23.   Auerbach's plexus of stomach of rats fed on the ' stock ' diet. × 280.

  ,,   24, 25, and 26.   Auerbach's plexus of stomach of rats fed on the ' tapioca diet ' for 9, 12, and 14 months, respectively.   × 280.

  ,,   27, 28, and 29.   Auerbach's plexus of stomach of rats fed on the ' cheap Madrassi diet ' for 9, 14, and 16 months, respectively. × 280.

The superficial mucosal erosions formed by the breaking down of one or more of the lymphoid follicles tend to heal rapidly and may not leave a trace. Conceivably a number of such follicles rupturing close together might coalesce into an ulcer which would become chronic on account of its size and the continued action of the gastric juice. One reason why ulcer is confined largely to the pyloric antrum and duodenum may be the greater accumulation of lymphoid elements in these regions and the possibility of multiple follicular abcesses coalescing to form one large ulcer. Miller (1906) has given a closely similar description of the early phases of a peptic ulcer.

The pathological changes found in the Auerbach's plexus are interesting in relation to the neurogenic theory of the genesis of peptic ulcer. Most of the animals fed on the deficient diets showed degenerative changes in the ganglion cells before any mucosal infiltration was noticed. From a study of serial sections in animals killed after various periods of deficient feeding, it was evident that the earlier pathological lesions in the nerve cells were degenerative; in the later stages, however, the plexus shared with the mucosa the general inflammatory change. The lesions of Auerbach's plexus reported here closely resemble those described by Stöhr (1932) in stomachs re-sected for ulcer.

The significance of changes in Auerbach's plexus in relation to peptic ulcer is obscure, as the exact function of the plexus is not clearly understood. It is generally agreed that the plexuses of Auerbach and Meissner act as local nerve centres for the gastro-intestinal tract. Alvarez (1928, 1929) believes that the centre of control of muscle movement and secretion lies in the muscle wall itself and the complicated impulses of the vagus and sympathetic are co-ordinated in Auerbach's plexus. Possibly degeneration of the plexus may lead to spasm, hypersecretion, and abnormality in function of the delicate mechanism of the pyloric antrum and pylorus. Evidence in support of this hypothesis has been brought forward by several workers who have studied cardio-spasm and Hirschsprung's disease. Etzel (1937) investigated cases of cardio-spasm occurring among the poorer people of Brazil and described changes in Auerbach's plexus in the œsophagus closely similar to those observed by us. Robertson and Kernohan (1938) reported similar changes in the myenteric plexus in Hirschsprung's disease.

McCarrison (1921) found degenerative changes in the myenteric plexus in animals (monkeys and pigeons) fed on deficient diets. These changes were frequently associated with motor imbalance, spasm, and intussusception. The fact that two dogs fed on the 'tapioca diet' died of intussusception is of interest in connection with the above findings. Similar observations were also made by Magee, Anderson and McCallum (1929) with cavies fed on deficient diets.

The frequency of intussusception in adults in Travancore was pointed out by Orr (1932) and it was suggested that an overaction of the vagus might be the cause.

In this connection mention may be made of the theory which associates peptic ulcer in Europeans with nervous strain and anxiety, leading to hyperperistalsis, pyloro-spasm, and hypersecretion.

Morton (1934) observed that when pyloric dysfunction was produced by encircling the pylorus with a ring of living jejunal muscle, inflammatory changes took place in the mucosa of the stomach and duodenum. Superficial erosions,

infiltration with leucocytes and plasma cells, congestion of the capillaries, œdema and fibrosis were noted. There was also increased glandular and epithelial activity and hypertrophy of the lymph follicles. This picture closely resembles that described in this paper and labelled chronic gastritis.

The plexus changes observed in our experimental animals were presumably the result of diet deficiency, and it is probable that the very similar changes in the human specimens were due to the same cause. The present investigation does not, however, indicate what specific deficiency or deficiencies in the diet may be held responsible. One of us (Radhakrishna Rao, 1936) has described similar changes in the Gasserian ganglion in animals fed on diets deficient in vitamins A and B_1. Similar but less marked changes were found in Auerbach's plexus in rats and rabbits fed on vitamin-A deficient diets (Radhakrishna Rao, 1939).

We have shown that degenerative changes in the plexus occur in cases of peptic ulcer and also in cases likely to develop ulcer at a later stage. Chronic inflammation of the mucosa accompanies or follows these lesions. Such inflammatory changes have been observed in individuals living on rice or tapioca diets not suspected of gastro-duodenal lesions. In rats and dogs fed on ill-balanced rice or tapioca diets similar changes in the plexus and less marked changes in the mucosa of the stomach and duodenum have been noted. In the North Indian specimens examined, inflammatory changes in the mucosa, which in the other clinical and experimental specimens were found in combination with plexus degeneration. were not present. While the observations recorded are suggestive, we do not at this stage wish to draw any definite conclusions about the ætiology of peptic ulcer in South India. It is to be noted that we have not been able to produce peptic ulcer in animals by deficient feeding.

SUMMARY.

1. The high incidence of peptic ulcer in South India is described. An attempt is made to investigate its pathogenesis.

2. Histological study was made of the changes in the stomach and duodenum occurring in cases of gastric and duodenal ulcer, in human controls, and animals fed on deficient diets.

3. In cases of peptic ulcer evidences of chronic gastritis or duodenitis and degenerative changes in Auerbach's plexus were observed in the stomach or duodenum at a considerable distance from the ulcer. Similar changes were also noted in patients with subjective symptoms of ulcer in whom no ulcer was found at operation.

4. Study of post-mortem material from subjects in North and South India respectively in whom there was no reason to suspect gastro-duodenal lesions showed that the stomach and duodenum in the former· were normal in histological appearance, while, in some of the latter, lesions in the mucosa and intra-mural nerve plexus, similar to those found in cases of ulcer, were present.

5. Degeneration in Auerbach's plexus. accompanied by some changes in the mucosa, has been consistently observed in' rats and dogs fed on diets resembling those consumed by the poorer classes in the Madras Presidency and Travancore (South India).

6. The significance of the above changes in relation to peptic ulcer is discussed.

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SEX-HORMONE ACTIVITIES OF ARTOSTENONE DERIVATIVES.

Part I.

ACTION OF ARTOSTERONE ON SEXUALLY IMMATURE MALE RATS.

BY

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SINCE the isolation of the male sex-hormone androsterone in the crystalline condition from human urine by Butenandt and his co-workers (1932, 1934) and its preparation from epi-dihydro-cholesterol in the laboratory by Ruzicka *et al.* (1934), extensive studies on sterols and sex-hormones have been made.

Recent observations that sterols other than ergosterol can be made to develop antirachitic properties (Windaus *et al.*, 1935, 1936, 1937) and investigations on the transformation of sterols into male as well as female sex-hormones have added greatly to the interest in this line and have led to the search for new sterols and sterol-derivatives in the plant and animal kingdoms.

In previous communications (Nath, 1937*a, b, c*) it has been shown that artostenone. the new stenone which has been isolated from the Indian summer fruit, *Artocarpus integrifolia,* is almost similar to other sterols in constitution, thus possessing. like sterols and sex-hormones. four condensed rings in a molecule. This has recently been substantiated (Nath and Mukherjee, 1939) by crystallographic investigations by means of *X*-rays, the results of which will shortly be published elsewhere.

It has previously been found that unlike all other sterols it contains no hydroxyl group on C_3 in ring I, but has a keto group on C_{12} in ring III (Nath, *loc. cit.*).

Having gained an insight into the internal structure of the artostenone mole-cule which resembles more or less the mother substances of sex-hormones, it was thought desirable to prepare, by elimination of the side chain, etc., some of its derivatives which might be similar to androsterone or some compound related to this, in their activities towards the development of the secondary sex organs in animals.

Working on the principle of Ruzicka *et al.* (*loc. cit.*) with some modifications, it has been found possible to prepare from artostenone, a hydroxyketone, melting at 172°C. to 173°C., and the name ' artosterone ' has been proposed for the substance (Nath, 1939). Details of the methods of procedure and of the chemical and physical properties of this newly prepared substance will shortly be published. It is to be recorded here that the melting point of artostenone, its mother substance, is 109°C. (Nath, 1937*d*), that of androsterone, the preparation showing male-hormone action, being 174·5°C. (Ruzicka *et al.*, *loc. cit.*).

With a view to ascertain whether ' artosterone ' has any physiological acti-vity, a series of investigations have been undertaken on different lines on different animals, the present communication representing the results of the study of its activity on sexually immature male rats.

Detection and assay of androgens (male hormones) by means of the prostate reaction were first developed by Moore *et al.* (1930). They found that a perfectly normal rat prostate can be regenerated in 20 days even on a daily injection of a very small dose (0·1 capon unit) of testis-tissue concentrate, in rats castrated about three months previously. They also observed a marked influence on prostate development in young rats, sexually immature.

Laqueur *et al.* (1931, 1934, 1935) used the method of seminal-vesicles reaction to estimate the activity of androgenic substances. This method was also adopted by Ogata and Hirano (1934) in determining the rat unit of a substance possessing male-hormone activity. Wang and Wu (1933) and Tschopp (1935), however, have shown that the seminal-vesicles test cannot be taken as specific for male hormones. Tschopp found that crystalline theelin, when injected in daily doses of 50 γ to 100 γ for 21 days, can stimulate the growth of seminal vesicles to an even greater degree than the same or higher doses of crystalline androsterone.

Korenchevsky and his associates (1932*a*, *b*, 1933*a*) have made extensive investigations on the effects of testicular hormones on rats, both castrated and sexually immature, by the method of weight-increase of the secondary sex organs, especially the prostate and seminal vesicles taken together. The effects of andro-genic substances on other organs and glands as well, have also been recorded. Koren-chevsky (1932*a*) recommended that, for comparative studies, rats should be selected from the same litter and made the following statement : " . . . for the simple assay of testicular hormones two rats as controls and two rats for injection are sufficient to obtain accurate information with regard to the positive influence of the extract ". Korenchevsky and Dennison (1935) actually prefer to take the percentage increase of the weight of prostate and seminal vesicles together to that of the prostate alone ; for they are of opinion that the combined

prostate and seminal-vesicles reaction represents the 'whole male sexual activity', while the prostate reaction is an indication of 'comb-growth' activity. It can be seen from their results that for doses of 200 γ, 450 γ, 600 γ, and 900 γ of androsterone, percentage increases in the combined weight of prostate and seminal vesicles are 46, 110, 145, and 221 or when calculated per 200 γ of androsterone are 46, 48, 48, and 49, respectively. The rat unit (R. U.) has been recommended as the minimum daily dose which, when injected twice a day during seven consecutive days into castrated rats, will produce an average increase of 40 per cent in the weight of the prostate plus seminal vesicles, as compared with untreated castrated litter-mates. Thus 170 γ androsterone has been suggested as one R. U., one capon unit (C. U.) varying from 150 γ to 200 γ of androsterone.

The present investigation deals with the effect of artosterone on the development of the secondary sex organs (especially the prostate and seminal vesicles), the endocrine glands, such as thymus and thyroid, and other important organs, such as liver, spleen, heart, and kidneys, of sexually immature male rats as compared with the development of those of the normal litter-mates as controls. A very small quantity of substance (artosterone) was available at the outset and the number of young rats at our disposal was not very great. These were sufficient, however, as recommended by Korenchevsky (1932a) to obtain accurate information on the androgenic properties of the substance.

Detailed investigations on the assay of artosterone by prostate and seminal vesicles development of castrated male rats and by comb-growth of capons, etc., are in progress.

Technique.

In each set of experiment there were at least two rats for injection of the same dose of androgenic substance and two as controls, all the four being selected from the same litter. The experiments were begun with rats of about one month old, weighing from 67 g. to 82 g. Doses of 50 γ and 500 γ of artosterone were injected daily and this was continued for 21 days.

In preparing the oily solution of the substance, it was dissolved in requisite amount in alcohol which was then poured into the calculated amount of warm olive oil. Alcohol was then removed as completely as possible *in vacuo* at 75°C. to 80°C.

Regarding the quantity of solution which should be used per injection, it was pointed out by Korenchevsky *et al.* (1932) that when more than 0·2 c.c. of oily solution was injected per day subcutaneously, on autopsy there was found some unabsorbed oil in the subcutaneous tissue at the place where the injections had more recently been made. In order that there may be proper absorption of the oil in the system we have adopted the method of intramuscular and subcutaneous injections alternately, and the daily injection of 0·2 c.c. solution was the maximum amount used.

The method which has generally been adopted for controlling animals by the previous investigators, is that they are allowed to remain as untreated or uninjected, and injections have only been given to the experimental ones.

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The method which has generally been adopted for controlling animals by the previous investigators, is that they are allowed to remain as untreated or uninjected, and injections have only been given to the experimental ones.

It has appeared advisable to us to inject the same amount of olive oil without artosterone in control rats to avoid any error due to the stimulating effect of pricking with the needle or any action of the oil itself and this has been done in the case of all controls.

Regarding the diet of the experimental rats, we preferred using the usual diet (i.e., whole-wheat flour and milk in equal quantities supplemented with cod-liver oil and vegetable juice) to artificially prepared ones. ·

At the end of the experimental period the animals were weighed and then killed by chloroform. On dissection the organs were separated as far as possible from the adhering fat and foreign tissue and plunged immediately for fixation and preservation in Bouin's fixative (modified by Allen) which consists of saturated solution of picric acid 75 c.c., formalin 15 c.c., glacial acetic acid 10 c.c., and urea 1 g.

The retroperitoneal fat which gives a quantitative measure of the deposition of body fat in rats was weighed while fresh, without further treatment.

The possibilities of sources of error which may affect the result, if the weights of the organs are taken immediately after dissection, without using any fixative solution, are as follows :—

(a) Degree of removal of water from the organs in the experimental animals and controls may not be uniform.

(b) With prolonged exposure to the atmospheric air while dissecting and weighing, some parts may undergo some degree of decomposition.

(c) Fresh organs may adhere to the weighing bottle, thus causing slight loss while removing from it.

(d) Separation of the adhering fat and foreign tissues are not so easy and complete as after fixation.

(e) As all the experimental rats have to be killed on the same day, the task becomes an impracticable one if the weight of all the organs has to be taken, while fresh.

For histological studies, dehydration of the fixed and hardened tissue, preparing blocks, cutting sections, staining and mounting, etc., were done in the usual way. Hæmatoxylin and eosin were used as the staining and counterstaining materials respectively.

The microphotographs of the sections were prepared with the help of Miflex (Zeiss) microphotographic apparatus.

EXPERIMENTAL.

The Table shows the average actual weights and those calculated per 200 g. of body-weight of the secondary sex organs, endocrine glands, and some other organs of rats injected with artosterone, compared with those of the organs of normal litter-mates (injected with the same amount of oil only) and their percentage changes.

PLATE VIII.
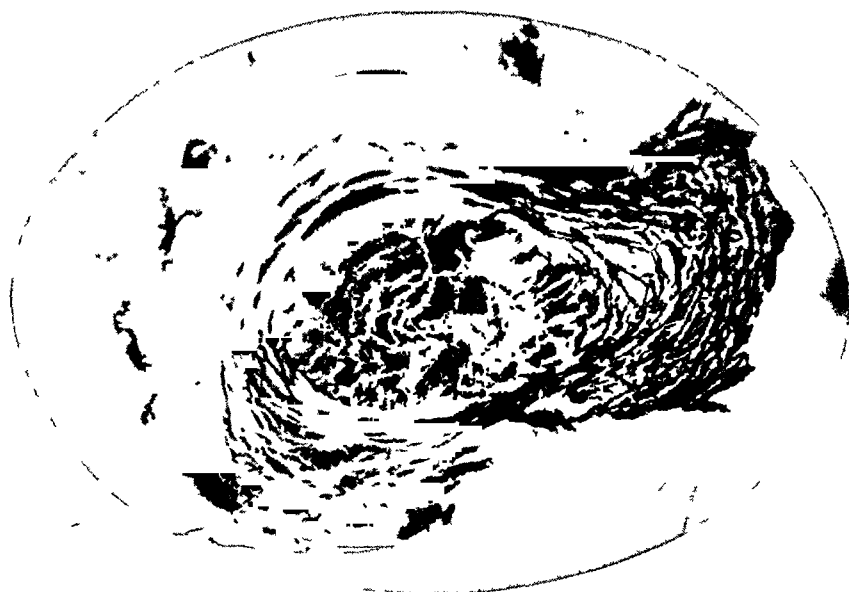
Fig. 1. Section of vas deferens of a control rat. × 70.
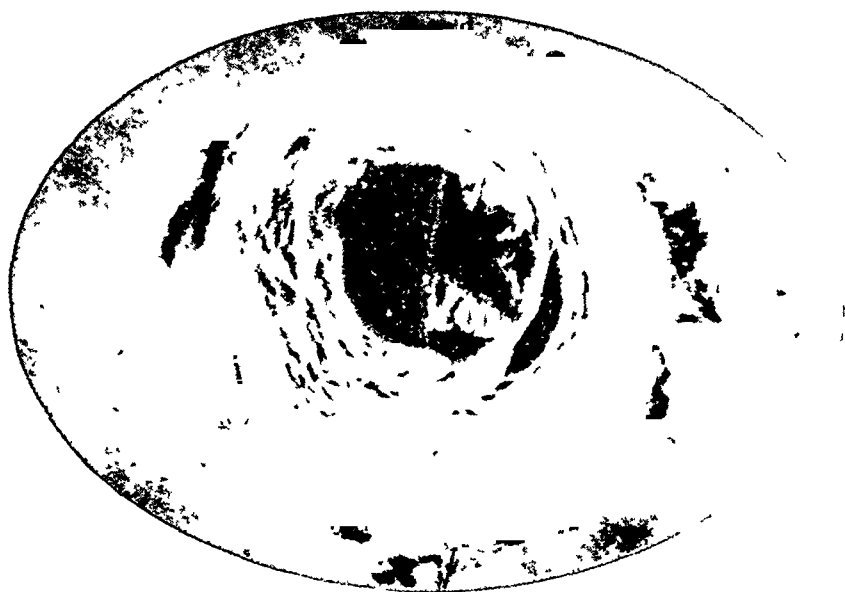
Fig. 2. Section of vas deferens of a rat injected with artosterone
(50 γ per day). × 70.

PLATE IX.

Spermatocytes.

Spermatids.

Basement
membrane.

Interstitial
cell.
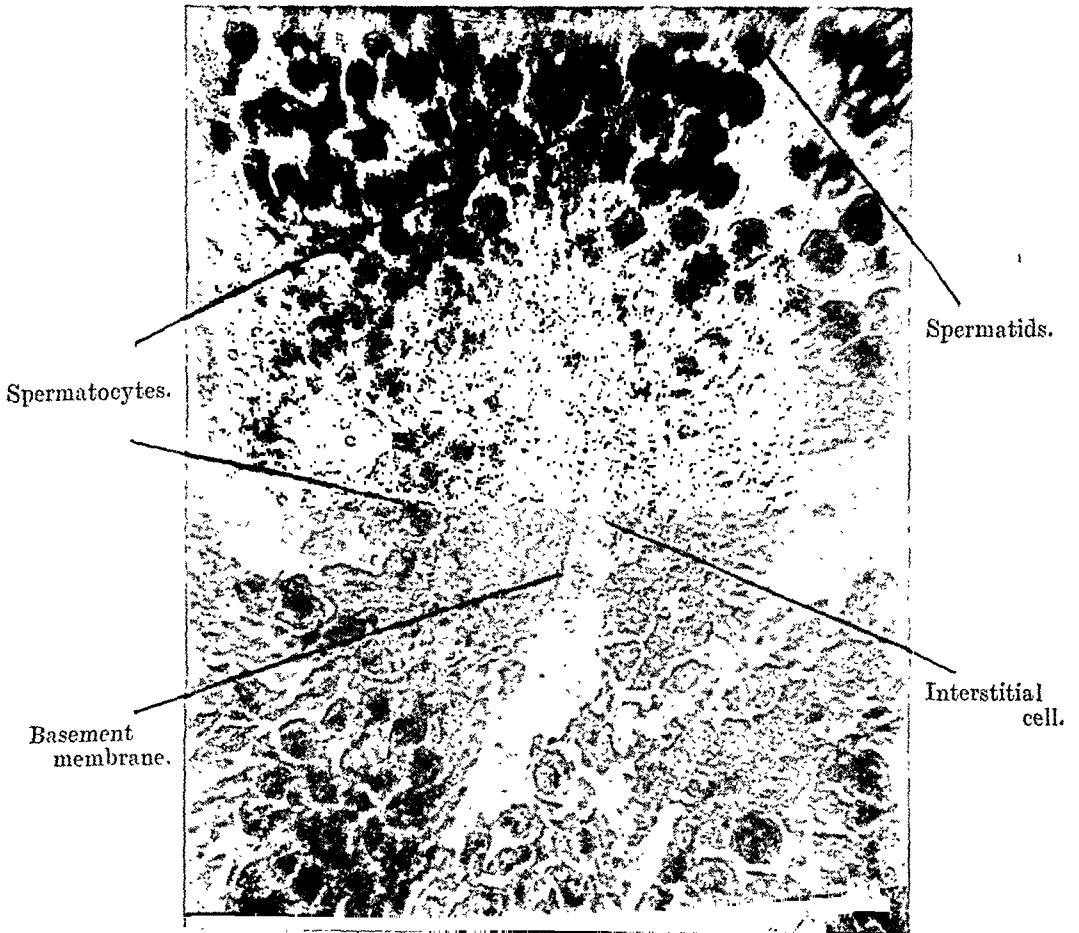
Fig. 3. Section from a testicle of a control rat. × 630.

Spermatocyte.

Spermatids.

Basement
membrane.

Interstitial
cell.
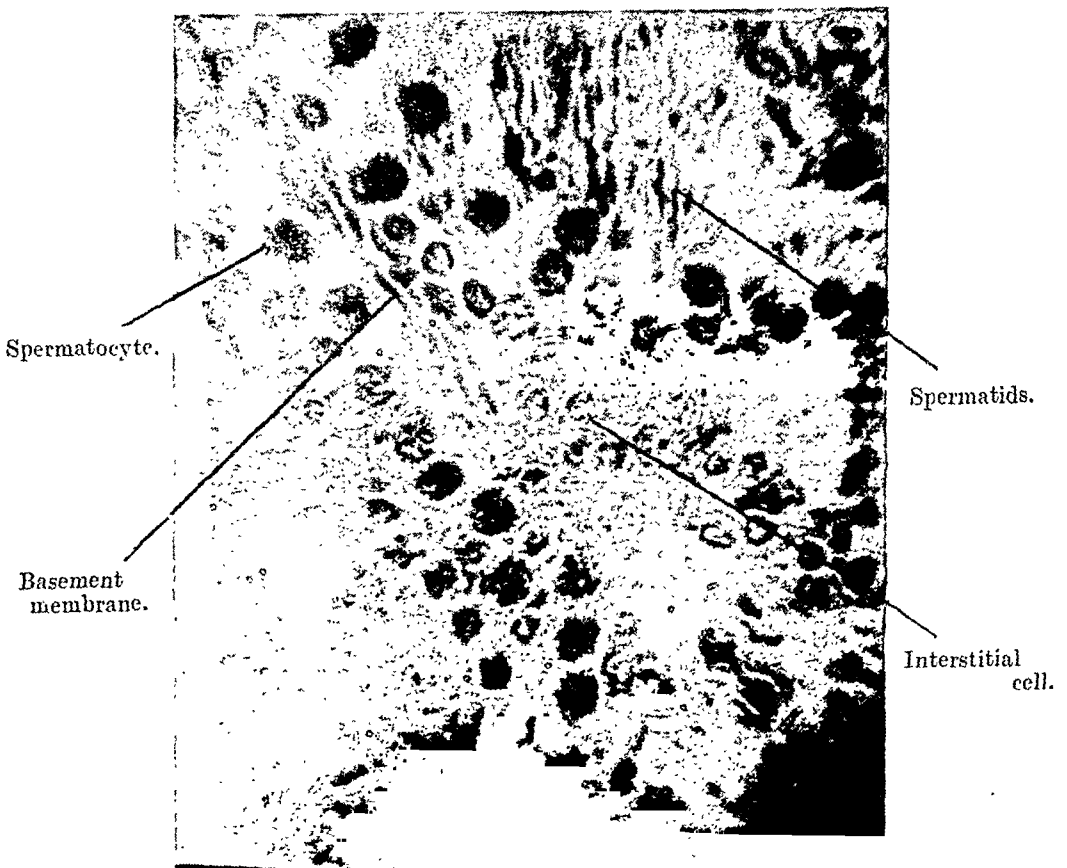
Fig. 4. Section from a testicle of a rat injected with artosterone
(50 γ per day). × 630.

TABLE.

Organs.	CONTROL RATS.		RATS INJECTED WITH ARTOSTERONE.			
			50 γ		500 γ	
	Actual (average).	Per 200 g. body-weight.	Actual (average).	Per 200 g. body-weight.	Actual (average).	Per 200 g. body-weight.
Prostate with seminal vesicles (mg.).	627	1,130	770	1,340	789	1,360
Per cent change ..	..	..	23	19	25	21
Penis (mg.)	56	101	64·5	112	59	103
Per cent change ..	..	..	15	11	5	2
Testes (g.)	1·55	2·80	1·48	2·58	1·52	2·66
Per cent change ..	..	..	−4	−7	−2	−4
Thymus (mg.) ..	186	335	159	276	179	314
Per cent change ..	..	..	−15	−17	−4	−6
Thyroid (mg.) ..	42·1	75·8	43·2	75·0	42·2	74·0
Per cent change ..	..	..	3	−1	0	−2
Liver (g.)	5·60	10·10	6·08	10·56	5·38	9·44
Per cent change ..	..	..	8	5	−4	−6·5
Spleen (g.)	0·47	0·84	0·52	0·90	0·46	0·81
Per cent change ..	..	..	11	8	−1·5	−4
Kidneys (g.)	0·75	1·35	0·84	1·46	0·84	1·45
Per cent change ..	..	..	11	8	11	8
Heart (g.)	0·47	0·84	0·42	0·72	0·41	0·72
Per cent change ..	..	..	−11	−14	−11	−14
Retroperitoneal fat (g.) ..	1·70	3·06	1·50	2·72	1·23	2·16
Per cent change ..	..	..	−12	−15	−28	−30
Average final body-weight (g.).	111	..	115	..	114	..
Average initial body-weight (g.).	77	..	74	..	75	..
Average change in body-weight (g.).	34	..	41	..	39	..

EFFECT ON THE SEX ORGANS.

Effect on the prostate and seminal vesicles.

It will be evident from the Table, that the injected animals, as compared with the controls, showed marked increase (19 per cent) in the weight of the prostate and seminal vesicles (calculated per 200 g. of body-weight) even with moderately small dose as 0·05 mg. (50 γ) per day. Korenchevsky, Dennison and Kohn-Speyer (1933a), working on the effects of testicular preparation on similar young rats of about one month old, obtained under almost identical conditions an increase of. 62 per cent of these organs, with a dose of 2D which is equivalent to 5 C. U. or about 1,000 γ of androsterone. This indicates definitely that the newly prepared substance 'artosterone' is highly potent in its androgenic activity.

Effect on the penis.

The influence on the penis was also interesting. With a small dose as 50 γ per day, the average increase in weight was found to be about 11 per cent when calculated per 200 g. of body-weight.

Effect on the testes.

The depressing effect on the testes, which the male sexual hormones possess in general, has been observed in this case also. Similar depression has been observed by Korenchevsky and his associates (1933b).

Effect on some other endocrine glands.

The figures in the Table show that there is marked acceleration in the rate of retrogression of the thymus. In rats receiving only 50 γ artosterone as daily injection, there has been noticed an average decrease of 17 per cent calculated per 200 g. body-weight. Korenchevsky and his co-workers (1933a, b) obtained the average decrease of 23 per cent and 25 per cent in weight of this gland with doses 1 and 10 R. U. of testicular hormone respectively for six litters of younger rats.

No appreciable effect on the thyroid was noted.

Effect on the liver and kidneys.

A slight increase in the average weights of the liver and kidneys was observed. Testicular hormone when injected into normal and castrated male rats had been found to cause some such increase in the weights of these organs by Korenchevsky et al. (1933b) and Korenchevsky and Dennison (1934).

Retroperitoneal fat.

A decrease in the retroperitoneal fat (15 per cent) was observed which is a typical action of substances possessing male-hormone activity. Similar decrease in the deposition of fat has also been observed by previous investigators of the effect of androgenic substances in rats (Korenchevsky et al., 1937a, b). It has also been observed by Korenchevsky (1936) that testosterone injection caused an improvement in the gain of body-weight of castrated rats without any increase in

the deposition of fat. From this they concluded that the gain in body-weight was due to the anabolic processes in building new tissues and not to an increase by deposition of fat.

HISTOLOGICAL INVESTIGATIONS.

Vas deferens.—Sections were cut more or less from the same part of the vas deferens in the controls as well as in the experimental rats, i.e., rats receiving artosterone injection. The most striking change in the structure of this organ is the opening up of the central lumen in the experimental rats, while in the controls the central lumen is closed and obliterated by the longitudinal folds of the mucous membrane. Complete development of epithelium and increase in the size of the lumen has been observed by Callow and Parkes (1935), as an effect of injecting androgens on castrated leghorn capons. there being observed total atrophy of this organ in controls. A similar result has also been obtained by Heller (1932).

The elastic fibres between the muscular bundles are more prominently seen in the controls, while the muscular bundles are found to be more prominent in the experimental animals. David *et al.* (1934) have also found such stimulating effect of sex hormones on the development of smooth muscle tissue of the secondary sex organs of both sexes.

Testes.—The spermatogenic cells of the seminiferous tubules of the controls are more numerous in number, closely packed and are found arranged in more numerous layers than in the case of the experimental ones where these cells are only a few layers thick and the primary spermatocyte stage is more pronounced in most of the tubules. The spermatids are more numerous in the control than in the case of the experimental animals but the spermatozoa are found in the tubules of both.

In the controls all types of cells, namely spermatogonia. primary and secondary spermatocytes, spermatids and spermatozoa, are seen in the several layers showing the gradual stages of development of spermatozoa from the spermatogonia.

In the case of the experimental rats the development of spermatozoa from the spermatogonia is hurried through two or three layers of cells. Wells and Moore (1936) and Baker and Johnson (1936) have observed as a result of injecting androgenic substances such acceleration in the rate of spermatozoön formation in the immature ground squirrel and in the sexually inactive ground squirrel respectively.

The absence of the gradual stages towards the formation of spermatozoa in the experimental rats can be explained in the following way : The rate of formation of spermatozoa is so much increased on account of the accelerating effect of artosterone that the primary spermatocytes cannot have sufficient time to pass through the normal gradual stages in the development of spermatozoa. Moreover, as the lumen of the vas deferens has been found open, it can be inferred that there is every possibility of the spermatozoa having been discharged from the seminiferous tubules.

The decrease in the weight of the testes which results from administration of androgenic substances may also be accounted for by the decrease in the number of the spermatogenic cells and also by the loss of spermatozoa from the seminiferous tubules.

All these observations confirm that artosterone, the newly prepared hydroxy-ketone, is highly androgenic.

Further investigations on the effect of this compound and its derivatives on the castrated and ovariectomized rats on the development of the secondary sex organs of sexually immature chicks and on the comb-growth of capons, etc., are in progress.

SUMMARY.

The changes produced by injection of an olive-oil solution of artosterone, the new hydroxy-ketone prepared from artostenone, for 21 days in the sexually immature male rats, have been studied, both by the method of percentage change of the organs (actual and calculated per 200 g. of body-weight) and by histological investigations.

An average increase in the weight of the prostate plus seminal vesicles of rats treated with artosterone compared with that of the control litter-mates even with a small dose of 50 γ per day, was found to be 19 per cent (calculated per 200 g. of body-weight).

There has also been observed (a) a depressing effect on the testes, (b) an acceleration in the rate of involution of the thymus, and (c) a stimulating effect on the kidneys.

Histological examination of the vas deferens shows that in the controls the mucous membrane is thrown into longitudinal folds, thus obliterating the central lumen, whereas in the experimental ones this is stretched up causing the opening of the central lumen.

In the testes, the spermatogenic cells in the seminiferous tubules are less in number and more prominently seen in most of the tubules in case of the experimental animals (i.e.. animals injected with artosterone) than in the case of controls where proliferation of the cells takes place, through gradual stages, at a slower speed.

Artosterone has thus been found to be highly androgenic in character.

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SEROLOGICAL REACTIONS IN KALA-AZAR : COMPLEMENT-FIXATION, FALSE WASSERMANN REACTION, AND HIGH ANTI-COMPLEMENTARY TITRE.

BY

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COMPLEMENT-FIXATION WITH WITEBSKY, KLINGENSTEIN AND KUHN (W. K. K.) ANTIGEN.*

IN work on leprosy with the same antigen by Greval, Lowe and Bose (1938) the only serious disturbing factor was found to be kala-azar. Further work with the antigen and sera from patients suffering from diseases other than leprosy has shown that the reaction is more ' specific ' for kala-azar than for leprosy or any other disease.

I. THE TECHNIQUE.

1. *The reagents.*

(i) *The hæmolytic system.*—This was prepared in accordance with require-ments of method No. 4 of the (British) Medical Research Committee's (1918) report on the Wassermann Test with the exception that red blood cell (r. b. c.) suspension was standardized by a method described by Greval *et al.* (1930). The minimal hæmolytic dose (m. h. d.) of the complement determined for the purpose of the Wassermann reaction, without antigen, was also used in this technique.

(ii) *The antigen dilution.*—A small volume of W. K. K. antigen (0·2 c.c.) was evaporated. The residue, free from the smell of benzol, was made into a uniform

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* The antigen was purchased from the Behring Werke, Germany, through their local agents, Havero Trading Co., Central Avenue, Calcutta.

suspension with saline twice the volume of the original liquid (0·4 c.c.). The suspension was the starting point as recommended by the makers of the antigen. From it were prepared dilutions of 1 in 10, 1 in 20, 1 in 30, 1 in 40, 1 in 50, and 1 in 60, and tested for anti-complementary activity with 1 m. h. d. of complement, thus :—

Tube :—	1	2	3	4	5
Antigen dilution, 1 vol. of :—	1 in 20	1 in 30	1 in 40	1 in 50	1 in 60
Complement dilution containing 1 m. h. d. in a volume :—	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.
Saline	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.
	Left at room temperature for ½ hour and at 37°C. for ½ hour.				
Suspension of sensitized r. b. c.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.
	Left at 37°C. for ½ hour.				

The volume employed was 0·25 c.c.

The strongest dilution of the antigen permitting a complete lysis of the r. b. c. suspension gave the titre of the anti-complementary activity of the antigen against 1 m. h. d. of complement and was taken for the test.

The titre varied from 1 in 40 to 1 in 60 with the sample of the antigen used. In work on leprosy referred to above it had varied from 1 in 10 to 1 in 20. Whether the figure was high or low, the dilutions corresponding to the titre gave the same results with known sera.

Most of the work was done with an old sample of the antigen of the titre of 1 in 60 (it had once given a titre of 1 in 20, the rise was most probably due to evaporation and concentration). Later, a new sample was obtained. Its titre was 1 in 40. Known sera diluted to determine endpoints of reaction gave the same reading with the two antigens used in different dilutions as indicated by their titres.

The titration of the antigen was not repeated in full every time the test was put up. A tube containing 1 dose of the antigen dilution found satisfactory on the previous occasion (usually a few days before) was put up with 1 m. h. d. of the complement and included as the antigen control in the rack. The titre rose extremely slowly and stayed constant for months.

Further, the quantity of the antigen in the dose used was found to be in excess of the needs of the reaction. No weakening of the reaction was noticed before the quantity was halved.

2. *The serum for the test.*

This was inactivated at 55°C. for 30 minutes and diluted 1 in 25.

3. *The test.*

(i) *An ensemble :—*

Tube :—	1 Serum control.	2 Test proper.
Serum dilution ,. ..	1 vol.	1 vol.
Antigen dilution	*Nil*	1 vol.
Saline	1 vol.	*Nil*
Complement dilution containing 2 m. h. d. in a volume.	1 vol.	1 vol.
	Left at room temperature for ½ hour and at 37°C. for ½ hour.	
Suspension of sensitized r. b. c. ..	1 vol.	1 vol.
	Left at 37°C. for ½ hour.	

The volume employed was 0·25 c.c.

(ii) *Reading of results.*—The antigen control and the serum control being free from inhibition of lysis, the inhibition in the other tube was read as follows :—

Complete inhibition of lysis   ..   .. = +   positive.

A trace of lysis   ..   ..   .. = T ⎫
More than a trace of lysis   ..   .. =. ± ⎬ doubtful.
                                                    ⎭

Lysis almost complete, only turbidity present, = ? — ⎫
   no coloured deposit, no difference in colour
   between this tube and the next.   ⎬ negative.
Lysis complete   ..   ..   .. = —   ⎭

For detecting traces of lysis and for making sure that no differences of colour existed between ? — and — the rack was left in the cold overnight.

(iii) *Reaction with a higher and a lower serum dilution.*—In the work on leprosy referred to above a serum dilution of 1 in 5 was used. This was done with a view to obtaining the maximum reaction and comparing the results with those of Witebsky, Klingenstein and Kuhn's (1931) technique which also aims at obtaining the maximum reaction.

In the present work six dilutions were used in the beginning : 1 in 5, 1 in 12·5, 1 in 25, 1 in 50, 1 in 100, and 1 in 200 (1 in 12·5 was obtained by doubling the volume of 1 in 25 in the test, i.e., two volumes of the dilution were used instead of one). It was soon found out that the positive reactions of all cases other than those of kala-azar (and leprosy, two cases of pulmonary tuberculosis and one case of dermal leishmaniasis) were abolished in a 1 in 25 dilution. The 1 in 25 standard was then adopted. In some cases the endpoint of the reaction was obtained by further dilution. A table summarizing the reactions of 132 cases is appended. In it reactions of all sera in a 1 in 25 dilution are given. Only some of them were put up in higher dilutions. A continuous line under the dilutions indicates that the dilutions were not put up.

After the compilation of the Table we have started putting up the sera as a routine in two dilutions : 1 in 25 and 1 in 100. Treated cases are put up in four dilutions : 1 in 25, 1 in 50, 1 in 100, and 1 in 200.

Incidentally, we vary the serum concentration and not the dose of the complement for two reasons : (*i*) the procedure is more economical inasmuch as less complement is needed and (*ii*) there is reason to believe that differences in complement-fixation· in general are brought out better with decreasing the serum than with increasing the complement.

(iv) *Paradoxical reaction.*—Working with 1 in 5 and 1 in 12·5 dilutions some sera were found to give a T reaction with the 1 in 5 dilution or even with the 1 in 12·5 dilution but a fully positive reaction with the higher dilutions. In all these sera the endpoint was not reached even in a 1 in 200 dilution. The interference with the full fixation of the complement in these cases was indicative of an excess of the reacting antibody. Attention was called to this reaction by the deepening of the trace of colour with the concentration of the serum. Direct hæmolytic action of a strong dilution was excluded.

II. Remarks and associated considerations.

1. *Special features.*

Unlike the original technique of Witebsky *et al.* (*loc. cit.*) in which their antigen was used, the present technique uses complement measured in terms of m. h. d. This feature is of particular interest in the tropics where the m. h. d. of the pooled complement varies from 1 in 30 or less during the hot months to 1 in 90 or more during the cool months. Besides, quantitative and repeatable readings of fixation can only be obtained with titrated complement.

The antigen dilution used is also standardized in terms of its anti-complementary activity. In our experience samples of the antigen have varied widely in this respect.

2. *The sensitiveness of the reaction.*

The sensitiveness is of a very high order. Like the strongly positive Wassermann reaction of secondary syphilis the reaction cannot be missed or masked by any error or irregularity which is likely to creep into a serological procedure. Because of this high degree of sensitiveness we have differentiated between + and T. grouping the latter with ±. For the same reason ?— and — have been grouped together.

3. *The specificity of the reaction.*

While there is ample excuse for using the term specificity in connection with the reaction of the sera of lepers with the antigen. because of the group relationship between the *M. tuberculosis* and *M. lepræ*, the use of the term in connection with the reaction in kala-azar is not justified. Undoubtedly the reaction is non-specific like the Wassermann reaction in syphilis and has probably the same immunological basis.

The diagnostic significance of the reaction is of a very high order. From the table summarizing the reactions of 132 cases (Indian, Anglo-Indian, adults and children of both sexes, Indian male adults predominating) it will be seen that not a single case diagnosed kala-azar has given a negative reaction. In category I out of 50 cases, 8 were in the wards of the Carmichael Hospital for Tropical Diseases and in them the parasite was found by puncture. The remaining 42 were diagnosed in the out-patient department by one of us (L. E. N.) by the aldehyde test and on clinical grounds. In category II all six cases were diagnosed by finding the parasite. Cases of other diseases, from the wards of this hospital, mostly febrile, excluding leprosy, have given negative reactions with the exception of a single case of pulmonary tuberculosis. This case was admitted to the hospital as a suspected case of kala-azar and was diagnosed tuberculosis radiologically in the first instance. Tubercle bacilli were not demonstrated in the sputum until after many examinations. Out of 25 cases of pulmonary tuberculosis from the Medical College Hospital, one case gave a positive reaction.

Included in the non-kala-azar series were several cases with a positive Wassermann reaction. They give a negative reaction with the technique described. In a previous series of 46 non-leprosy Wassermann positive cases, positive reaction was obtained in one with the technique then described (Greval, Lowe and Bose, *loc. cit.*) in which the serum dilution used was 1 in 5. It is doubted if with the present technique a similar occurrence will be possible. Besides, in the case giving the reaction with the other technique kala-azar, other forms of leishmaniasis and tuberculosis were not excluded.

Apparently, the physical basis of the reaction is a constituent of the serum which when sufficient in quantity also reacts with formaldehyde which besides reacting with sera from cases of kala-azar reacts with sera from leprosy and tuberculosis, more or less.

4. *The utility of the reaction.*

The Table summarizing the work gives six cases which were doubtful or negative with the formaldehyde test and fully positive with the reaction. Later, they were proved to be cases of kala-azar by sternum, liver. or spleen puncture. In two of these cases attempts were made to determine the endpoint of the reaction, which was found to be over 1 in 200 for the one and over 1 in 100 for the other. A strong reaction, therefore, would create a very strong presumption in favour of a diagnosis of kala-azar, if not establish it for all intents and purposes, even in the absence of a positive formaldehyde test.

The reaction can be performed with a portion of the serum obtained for the formaldehyde test. Only those sera which are negative, doubtful or weakly positive

with the latter reagent need be subjected to the reaction. Only when they are negative with the reaction also, need the sternum, liver, or spleen puncture be made.

The above deductions are based on the unmistakable indication given by the reaction in the diagnosis of the disease in six cases. When the number of such cases has increased their force will be enhanced.

We have not tested the reaction of many treated cases of kala-azar. A steady fall and a negative reaction after an apparent cure may indicate a real cure and freedom from relapses.

Diagnosis of dermal leishmaniasis and oriental sore are other possibilities. Of the former we have tested five cases, of the latter none so far.

The serum need not be fresh and may be received by post as in Wassermann reaction.

The only possibilities to be considered in the differential diagnosis of a case giving a positive reaction are (*i*) kala-azar, (*ii*) other forms of leishmaniasis, (*iii*) leprosy, and (*iv*) tuberculosis.

False positive Wassermann reaction in kala-azar.

Greval, Sen Gupta and Das (1938) have recently stated that several diseases other than syphilis are responsible for a positive Wassermann reaction (W. R.). Kala-azar is one of them. Including the one case described by them five cases have been studied in the Carmichael Hospital for Tropical Diseases, Calcutta. The following is a brief description :—

> *Case 1.* P. B., Bengalee Hindu female aged 23. Admitted on 30th August, 1937, for kala-azar. W. R. positive. Treated with Neostibosan. Improvement. W. R. negative on 28th September, 1937. No signs or history of syphilis and no anti-syphilitic treatment.

> *Case 2.* Z. K., Mohammedan female child aged 9. Admitted on 20th November, 1937, for relapse of kala-azar. Duration 2 years. W. R. positive. Treatment for kala-azar. W. R. doubtful. Died of acute bacillary dysentery. No signs or history of syphilis and no anti-syphilitic treatment.

> *Case 3.* S. B., Hindu female aged 40. Admitted on 24th February, 1938, for kala-azar. Duration 1 year. W. R. positive. A course of Solustibosan finished on 15th March, 1938. W. R. again positive. General improvement as a result of treatment. Fit for discharge on 22nd April, 1938. W. R. doubtful only. No signs or history of syphilis and no anti-syphilitic treatment.

> *Case 4.* A. R., Mohammedan male child aged 5. Admitted on 2nd May, 1938, for kala-azar. W. R. positive. On Solustibosan from 7th May, 1938, to 16th May, 1938. W. R. doubtful. General improvement. Discharged on 21st May, 1938, with completely negative W. R. No signs or history of syphilis and no anti-syphilitic treatment.

In these cases the W. R. changed from positive to doubtful or negative with the control of kala-azar. In the next case the reverse occurred.

> *Case 5.* S. C., Hindu male aged 45. Admitted on 12th June, 1938, for abdominal pain and alternating constipation and diarrhœa. On routine examination were found L. D. bodies and a doubtful W. R. No fever. A course of Solustibosan. Febrile reaction, later relief. W. R. positive before discharge.

Was the fifth case an early case in whom constituents responsible for the false W. R. had not yet fully developed when the blood was first tested ?

During the same period we have observed cases of kala-azar with signs and history of syphilis. Anti-syphilitic treatment has been given to one of them with good results.

In 40 cases treated in the hospital between 31st August, 1937, and 31st August, 1938, we found :—

	Number of cases.
False positive W. R.	4
True ,, ,, ...	3
(False) doubtful W. R.	15
Anti-complementary sera	5
Negative W. R.	13
TOTAL ..	40

The positive, doubtful, and anti-complementary rates are very much higher than the rates for an unselected hospital population given by Greval, Sen Gupta and Das (*loc. cit.*), recently.

HIGH ANTI-COMPLEMENTARY TITRE OF SERUM IN KALA-AZAR.

It is known that sera not only become anti-complementary as a result of bacterial growth, but also are occasionally found to be so to begin with, when freshly separated from freshly drawn blood. In our experience the extreme limit of this property of the serum is reached in kala-azar. In the list given under the last item, FALSE POSITIVE WASSERMANN REACTION, 1 serum in 8 was found anti-complementary. In some cases no reaction (W. R.) could be read until the serum was diluted 1 in 20 (instead of the usual 1 in 5). For the same period from the same hospital the general rate of the anti-complementary sera was 1 in 50.

In the sera of known kala-azar cases given in the Table at the end of this paper there were 7 anti-complementary sera out of 56, when diluted 1 in 5, again a rate of 1 in 8.

In reading the results of the Wassermann reaction we have been so much struck by the association of a high anti-complementary titre of sera with kala-azar that we suspect all cases from which sera of such a titre have been obtained to be cases of kala-azar.

Harrison (1931) refers to observations of Thomsen and Bjarnhjedinson made in 1910 to the effect 'that leper serum is very anti-complementary'. The anti-complementary titre of the sera from kala-azar cases is of a much higher order. Further, working with fresh sera, we have not found sera from lepers very anti-complementary.

In a 1 in 25 dilution of a serum, which we now use as a routine for testing for kala-azar, the anti-complementary nature of the serum is not apparent as a rule. Occasionally, however, even in this dilution a serum is found anti-complementary.

ACKNOWLEDGMENT.

TABLE.

Summarizing the reactions of 137 cases with W. K. K. antigen.

Categories.		Complement-fixation in serum dilutions.			
		1/25	1/50	1/100	1/200
I. Kala-azar cases—50					
Aldehyde test positive	4	+	+	+	+
	2	+	+	+	——
	6	+	+	——	——
	1	+	T	——	——
	37	+	——	——	——
	50				
II. Kala-azar cases—6					
Parasite found	1	+	——	——	——
Aldehyde test negative	1	+	+	+	——
	2	+	——	——	——
Aldehyde test doubtful	1	+	+	+	+
	1	+	+	+	±
	6				
III. Control cases—50		—	——	——	——
(Diseases other than kala-azar and leprosy, from the wards of Carmichael Hospital for Tropical Diseases.)					
IV. Control cases of pulmonary tuberculosis—25.					
(From the wards and out-patient department of Dr. A. C. Ukil, Medical College Hospital.)					
	1	+	+	±	—
	5	±	——	——	——
	19	—	——	——	——
	25				

TABLE—*concld.*

Categories.		Complement-fixation in serum dilutions.			
		1/25	1/50	1/100	1/200
V. A suspected case of kala-azar. No parasites found. Aldehyde test positive. Diagnosed pulmonary tuberculosis radiologically. Tubercle bacillus found after many examinations of sputum.		+	+	T	±
VI. Dermal leishmaniasis—5					
	1	+	T	? −	——
	1	T	±	−	——
	1	±	? −	−	——
	1	±			
	1	? −	−	−	——
	5				

SUMMARY.

1. A technique of complement-fixation with W. K. K. antigen in kala-azar has been described. Hæmolytic system of method No. 4 of the Wassermann Test of the (British) Medical Research Committee, maximum quantity of the antigen not interfering with 1 m. h. d. of complement, and a 1 in 25 or more dilution of the serum to be tested are required. The diagnostic value of the reaction is very high.

2. The serum from some cases of kala-azar gives a false positive Wassermann reaction.

3. The limit of the anti-complementary activity of the human serum appears to have been reached in some cases of kala-azar.

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Postscript.—

De Liddo (1939) reports positive reactions with the W. K. K. antigen in several morbid states and in healthy subjects.   Our experience is different.

—*S. D. S. G.*—14-6-1939.

ADDITIONAL REFERENCE.

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INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part VII.

FURTHER FIELD STUDIES AND CONTROLLED EXPERIMENTS.

BY

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IN a previous communication (Lal, Ahmad and Roy, 1938) it was pointed out that for the solution of the problem of epidemic dropsy from the preventive aspect, it was necessary to develop methods by which batches of mustard oil containing the deleterious substance could be detected and, if possible, the toxic factor could be isolated so that its nature and origin might be investigated. The first requirement for these investigations was the collection of sufficient quantities of oil which could be definitely associated with outbreaks of the disease. With this object in view a series of field investigations were undertaken wherever a suitable opportunity occurred. Incidentally, these investigations served to test deductively the mustard oil theory as propounded in a previous communication (Lal and Roy, 1937*a*). A brief description of each of these studies is given below :—

(1) *Khargpur outbreak* (period of investigation—15th August, 1937 to 24th August, 1937).—Khargpur is a small town on the B. N. Ry., about 72 miles west of Calcutta. It consists chiefly of the railway colony. The outbreak occurred during the latter part of July and in August. A short note on this outbreak has already been published (Lal, 1938). It is proposed here to give some further details.

A total of 124 cases involving 38 families occurred towards the last week of July and in August 1937. The epidemic was practically confined to the Traffic Quarters in the north-eastern part of the railway colony and to Panchberia, a village situated just outside the town (*vide* Diagram 1)*.

<div align="center">DIAGRAM 1.</div>

KHARGPUR

The traffic quarters & the adjoining Punchberia Village

VILLAGE PANCHBERIA

⚲ BISWAKARMA TEMPLE

I N D E X

■ *Affected family.*

■ *Unaffected family having an imported case in the family*

1,2,3, etc.The chronological order of development of 1st cases in the families

NURSERY

TEMPLE

PILGRIM SERAI

POINT QUARTER

MOORUM QUARRY

SWEEPERS QUARTER

RECREATION GROUND

PLATFORM   STATION BUILDING

CHURCH

EPIDEMIC DROPSY INQY. I.R.F.A. AUGᵗ. 1937.

Khargpur is said to have been free from the disease in previous years. The first patient, a woman, is said to have come sometime in June from Benares while still suffering from the disease. She lived with a family in the Traffic Quarters for about a fortnight when she, along with the rest of the family, left the station. In the third week of July, 12 out of 28 members of the Y. M. C. A. Hostel situated in another part of the town were affected. Soon after, a crop of cases appeared in the Traffic Quarters and in Panchberia and within four weeks 38 families were

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*Diagrams 1 and 3 are reproduced by kind permission of the Editor, *Calcutta Medical Journal*.

involved. The morbidity rate in the affected families was 43·5 per cent. On investigation the following points were brought out :—

The general clinical picture closely resembled those seen in our previous field studies and in the experimental subjects. In 31 instances œdema was the first symptom noticed, in 23 it was preceded by diarrhœa, and in one case there was history of fever. No heart lesions could be detected in 37 subjects. In 12 patients slight and in four moderate heart lesions were present. Pigmentation of the face was noticed in 18 patients. The distribution of cases according to age, sex, etc., corresponded to our previous experience. Children under the age of five altogether escaped; young adults were the worst sufferers. Male cases slightly exceeded the female ones, the former being 58 per cent of the total. However, the constitution of the population was not known, though one might expect a larger percentage of males than females in an industrial town like Khargpur. Cases amongst the Hindus were three times as many as those amongst the Mohammedans ; however, it may be mentioned in this connection that in the Traffic Quarters where the cases chiefly occurred, the population was predominantly Hindu, while in the village it was mostly Mohammedan. In accordance with our previous experience, the lower-middle class contributed a majority of the cases.

·It was found that rice supplies were derived from different sources. Thus, nine families used self-grown rice, one obtained their supply from various cultivators, four were regular customers of Panchberia mill retail shop, and sixteen purchased rice from other shops in the town. The usual custom was to throw away water in which rice was boiled ; only a few families did not conform to this practice.

Although the affected families in the Traffic Quarters were a mixed population, hailing from different provinces, they all used mustard oil for cooking purposes ; so also did those in Panchberia village. It may, in this connection, be mentioned that, while in previous years the Panchberia mill oil was stocked by certain shops in the market in the town, this arrangement had broken down some time before the epidemic on account of disagreement between the proprietor and the shopkeepers and the amount of oil pressed in the mill was greatly reduced and mostly sold to the people residing in the neighbourhood of the mill. Thirty-one of the affected families obtained their oil direct from the mill, one got their supply of oil pressed at the Panchberia mill through a grocer, and four through a hawker. In one instance both Panchberia mill oil and some other oil were consumed, and the source from which the hawker supplied the Y. M. C. A. Hostel could not be ascertained. It will thus be seen that in fact in all known instances, the source of supply of mustard oil was common to all the affected families, whether they lived in the railway colony or in the village. While we are not in a position to say whether some other articles of food like ' dhal ', spices, etc., were not obtained from a common source (probably they were in a majority of instances), in view of the previous experiences the Panchberia mill oil comes in for strong suspicion. In this connection it must also be remembered that the railway employees living in the Traffic Quarters being mostly Hindus and strangers to the locality had no common interests or social intercourse with the villagers who were mostly Mohammedans following their ancestral occupation of agriculture. This fact by itself would cast doubt on the infection theory through direct contact. As against the possibility of the first case

being the source of infection for subsequent cases, it may be stated that the family which moved into the house immediately after the patient and her associates had left the town, was not attacked. Moreover, there was a long interval of more than a month between the date the imported case left the town and the date of appearance of the first case in the Y. M. C. A. Hostel which, in any case, was not in direct communication with the family. Unfortunately, we were unable to obtain samples of oil (from the mill or the affected families) which had been consumed previous to the appearance of cases. A sample of oil (No. 3), collected by the local Sanitary Inspector from a shop which had supplied Panchberia mill oil to some affected · families, proved non-toxic in a feeding experiment on human volunteers.

(2) *Garpar outbreak* (period of investigation—24th August, 1937 to 28th August, 1937).—This outbreak occurred about the same time as the Khargpur epidemic and like it, it furnishes another instance of grouping of cases round a mill supplying oil direct to the consumers. Garpar area is situated in Ward No. 4 of Calcutta. The epidemic was brought to our notice through the courtesy of the Health Officer. Fifty-two persons were attacked within a period of three weeks involving 12 families. The morbidity rate in the affected families was 45·2 per cent. The affected houses were situated round about the mill, but they were not contiguous. The residents of the area were almost entirely Bengalee Hindus following clerical and allied professions.

The clinical and epidemiological features were similar to those observed in epidemics already described. The point of interest was the common source of mustard oil which was obtained directly from the mill by all the affected families. Of the 23 unaffected families in the neighbourhood which were investigated only two used oil from this source. During this investigation an interesting point came to our notice. Two brothers with their families lived in the same house but messed separately. The members of the two families came into intimate contact with one another and yet there were four cases out of a total of 14 members (12 adults and two children under five) in the family which used oil from the mill and none in the six members of the other family which obtained oil from another source. The affected family usually purchased 2½ seers of oil at a time and this amount sufficed for about 10 days. The supply from which a sample was obtained (No. 12) was purchased directly from the mill on 2nd August, 1937. The first case appeared sometime during the week. The association of this particular supply with the disease is therefore not established, although it is probable that it belonged to the same batch as the one from which previous supplies were obtained.

(3) *A family outbreak at Kidderpore* (period of investigation—8th January, 1938 to 11th January, 1938).—This was a small outbreak involving the household of a Sindhi merchant at Kidderpore. There were 14 members, but with regard to their messing they could be divided into three groups as follows :—

    I.   House-owner, his wife, and three children.

    II. Six servants, three males and three females.

    III. Two shop assistants and the motor-car driver.

All the six members of the group II suffered from the disease but none others. The history of this outbreak is briefly as follows :—

· During the ' Ramzan ' in November 1937, all the members of the house received their food from the master's kitchen. After the fasting month was over, the members

again separated into three groups for purposes of feeding. The master's food was cooked separately by the servants, though occasionally the former took one or two dishes from the servants' kitchen. Members of group I took only one meal of rice a day and little or no mustard oil, the culinary fat used being mostly 'ghee'.

The servants cooked separately for themselves and were accustomed to the usual Bengalee diet, viz., rice, 'dhal', fish, and vegetables, the cooking-fat being exclusively mustard oil.

After the 'Ramzan' was over, on about the 5th December, 1937, the servants purchased five seers of mustard oil (sample No. 15) from a shop in Kidderpore. About a week later, when each servant had consumed about eight ounces of the oil, they developed gastro-intestinal disturbance and after another week or ten days, all the servants of this group developed œdema of legs and other symptoms which were diagnosed as epidemic dropsy by the family physician. Of the three persons of this group who were present at the time of investigation two exhibited œdema of legs, and one had heart lesions. According to the master of the house one woman servant had left the service because a well-known eye-specialist of Calcutta who had been consulted had diagnosed her as a case of glaucoma and advised operation. He had received the information that the woman had subsequently lost her sight. The third group took their meals in different hotels.

While the distribution of cases is so definitely related to messing arrangements, it is interesting to note that prolonged intimate contact had no significance in regard to the distribution of the disease. The sleeping arrangements in the household are shown in Diagram 2. The three children, aged eight, six and four, slept on the same platform with the maid-servants before and for about two weeks after the servants were attacked with epidemic dropsy, but all the children remained unaffected. The cooks and servants continued with their usual duties which required prolonged and intimate contact between the diseased and the healthy groups but the disease remained strictly confined to the members of group II. Here, of course, no one article of food can be singled out as the source of trouble, but this outbreak offers a good example in which intimate personal contact can be ruled out as a factor in the ætiology of the disease.

(4) *Rangpur outbreak* (period of investigation—16th January, 1938 to 19th January, 1938).—Rangpur is a municipal town in North Bengal and headquarters of the district of the same name. It is connected with the E. B. Ry. system. The total area of the town is about 10 sq. miles and the total population 20,749, according to the last census. The epidemic occurred towards the latter part of December 1937 and early in January 1938. A short note on this outbreak has already been published (Lal, *loc. cit.*). It is proposed to give some more details here.

Towards the beginning of the third week of December 1937, two Hindu and two Mohammedan families in four different wards of the municipality were almost simultaneously affected. Next week no new family was attacked but a week later fourteen more families were involved, to be followed in another week's time by six more families, after which the epidemic completely died down. Altogether 65 cases occurred in these 24 families. A striking feature of the epidemic was the

DIAGRAM 2.

KIDDERPORE FAMILY OUTBREAK

SLEEPING ARRANGEMENTS

FLOOR

GROUND FLOOR

COURT YARD

Servants Quarters

Ground floor

FIRST FLOOR
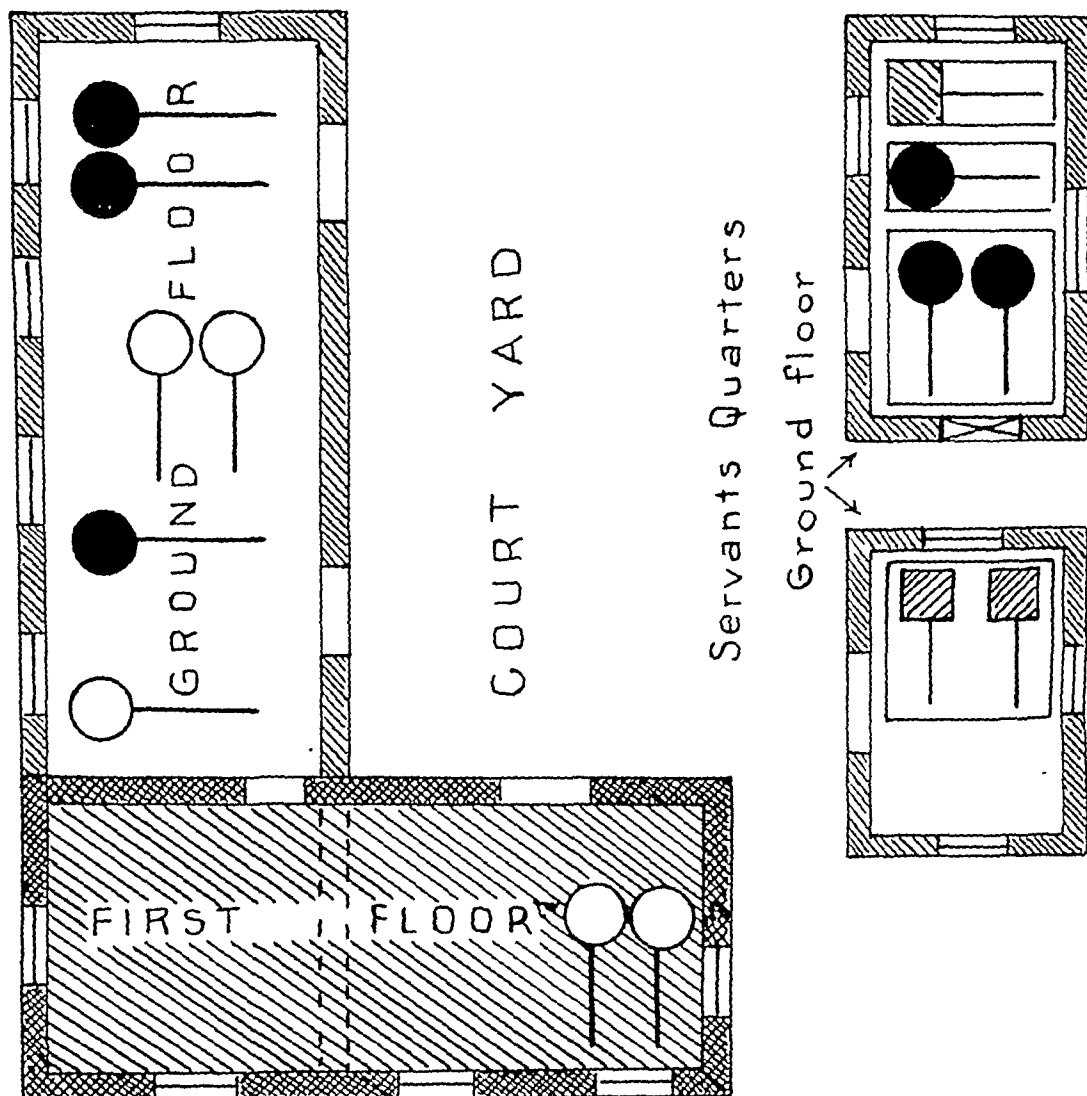
○——	MASTER	*Unaffected.*
●——	SERVANTS	*Affected.*
▨——	SERVANTS	*Unaffected.*

grouping of the affected families.   Their spatial distribution has been shown in
Diagram 3.   Group I in the southern part of the town consisted of Mohammedan
cultivators, and one of the first families to be affected during the outbreak belonged
to this group.   On the south-eastern side of the town may be seen the second group
of affected families.   These were mostly fishermen, both Hindu and Mohammedan,
living in a 'bustee' around the fish market.   This group was affected during the

DIAGRAM 3.

(EPIDEMIC DROPSY INQ^Y. I.R.F.A. JAN.1938.)
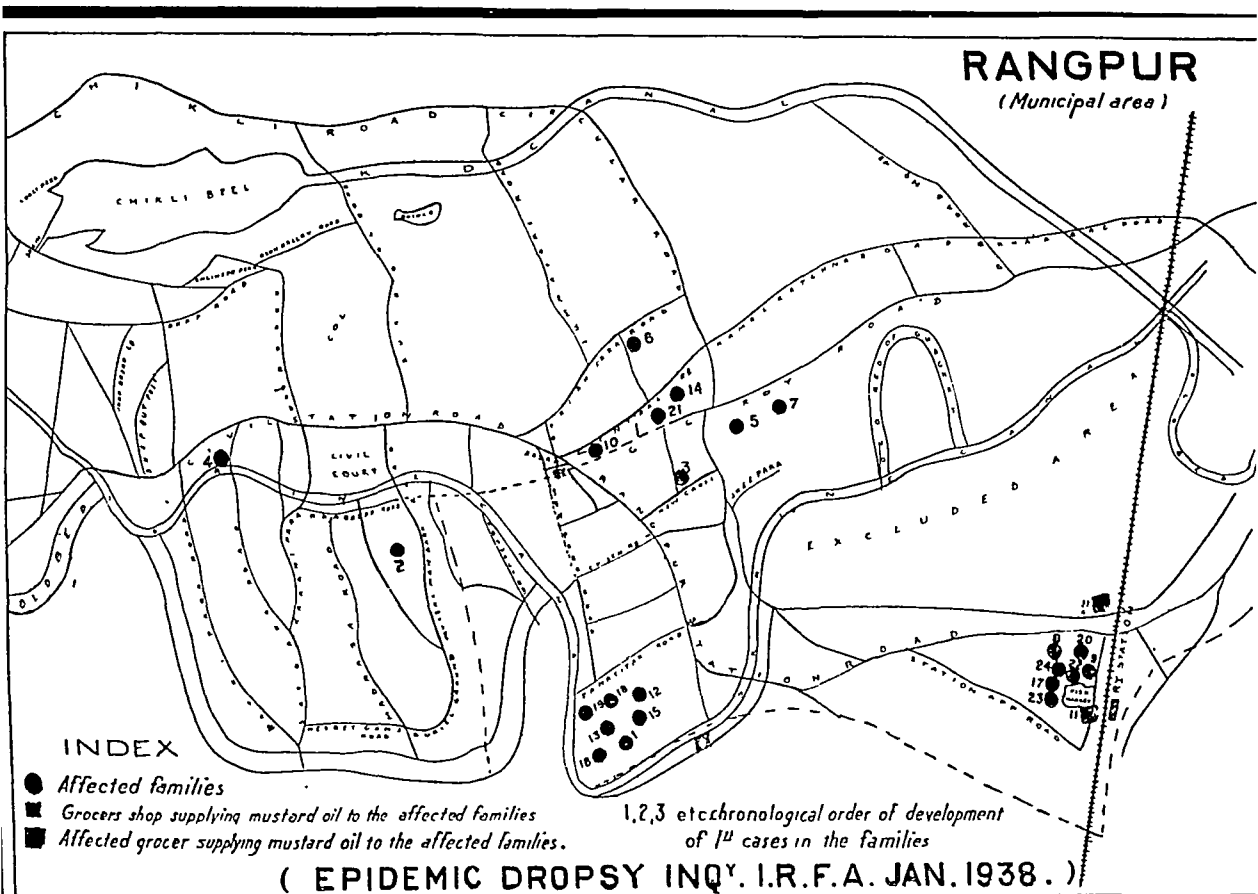
height of the epidemic.   The distribution of the third group of affected families
was not so compact as that of the second one.   They were scattered in the central
part of the town.   The chronological order of development of the first cases in these
families is shown in the diagram.   The signs and symptoms were :—

    1.   Intestinal disturbance and/or fever followed within a few days by œdema
           of both legs.

2. The characteristic œdema, pitting on pressure, almost completely disappearing in the mornings only to reappear in the evenings. In two cases, the characteristic flush in the swollen legs.

3. Dyspnœa with exertion in a number of cases.

4. No marked calf tenderness in any of the cases, nor any evidence of peripheral neuritis or of urinary trouble.

The general distribution of the cases with regard to age, sex, religion, etc., was in conformity with our previous findings (Lal and Roy, 1937*a*).

On investigation it was found that the rice market, locally known as 'amode', was the principal source of rice supply for the municipal area. Here the cultivators brought their hand-pounded rice and offered it for sale. There was also some amount of milled rice for sale, but the quantity was comparatively small. No group of affected families could be traced to any particular source of rice supply. Besides, the group of Mohammedan cultivators, both affected and unaffected, daily husked their own paddy stored in their houses, by the 'dhenki' method. This paddy had been recently harvested. In all the affected families, the custom was to throw away the rice-water.

With regard to mustard oil, the general custom in the locality was to buy a 'bhatful' (about 2 seers) of oil at a time from 'kolus' who sold this commodity from door to door. It would at first sight appear that there was no common source of mustard oil supply for the affected families. On investigation, it was found that these hawkers, instead of selling 'ghani' oil, were purveyors of mill oil which they used to purchase from the local merchants. The house-holder did not necessarily always buy from the same hawker though usually one fixed man was the principal supplier. These customs made it extremely difficult to test if any particular brand or consignment of mustard oil was responsible for the outbreak. However, in the fish market area, where about 50 families lived under 'bustee' conditions, 15 persons involving eight families were attacked during the course of less than two weeks and all of them had a common source of oil supply, viz., a grocer living in the 'bustee'. His family was amongst those affected. His brother, also a grocer, stocked the same brand and consignment of oil and there were some cases amongst his clients as well. The available oil of that particular supply (hereafter called Rangpur oil or sample No. 20) from both these grocers was collected and successfully used in a human feeding experiment referred to in a later part of this communication.

(5) *Alamdanga outbreak (Dt. Nadia)* (period of investigation 21st January, 1938 to 25th January, 1938).—There was no general outbreak of the disease in the villages investigated, but individual families were affected. The details are shown in Table I.

It will be noticed that the number of cases is very small. Besides, the signs of the disease at the time of investigation were not very marked, thus making the diagnosis rather uncertain. However, a feature of interest in connection with this epidemic was that the grocers supplying mustard oil to the affected families in different villages stocked amongst other brands, one particular brand which for

TABLE I.

Number of cases in the different villages investigated.

Name of village.		Total population.	Number of families affected.	Total cases.
Alamdanga	..	400	1	3
Bandabil	..	1,000	4	10
Bademaji	..	1,000	3	7
Benodepur	..	1,000	2	2
Jamjami	..	200	2	6
Nrishinghapur	..	500	3	4

short may be called 'H' brand. At village Bandabil this oil, in its original container, was found to be in use by an affected house and the whole quantity of the available oil was collected (No. 19). The epidemiological history of this oil is given below.

Three closely related 'kolu' families lived in one house. Two of them were affected with epidemic dropsy, the third, consisting of a single woman, had escaped. This woman used to buy mustard oil in original containers (about 20 seers in each) from a fixed shop in Alamdanga. She used to sell it from door to door. The two affected families in the house got the supply for the last few months only from her and from no other source, and this particular batch of oil was consumed by the families for about one week before the symptoms started. It proved mildly toxic when tested on human volunteers.

(6) *Manirampur outbreak (near Barrackpore)* (period of investigation—27th June, 1938 to 4th July, 1938).—The outbreak was mainly confined to a 'para' where about 40 Brahmin families lived. The first case was reported towards the end of May 1938, and further cases developed during the following month towards the end of which the investigation was undertaken. Altogether 98 cases occurred involving 22 families. The salient features of the outbreak were as follows :—

One person (K. M. C.) residing in the affected part of the village was keeping a grocer's shop. He belonged to the same social and economic class as the majority of the villagers living in that area. Of the 22 affected families, 18 obtained both their rice and mustard oil supplies regularly from K. M. C.'s shop and the remaining four families got only their mustard oil and not rice from that shop. The shopkeeper had different varieties both of sun-dried and parboiled rice, but he had only one variety of mustard oil to offer for sale, viz., 'G. M.' oil. The consumption of this particular oil was a factor common to all the affected families. There were nine unaffected families interspersed between the affected ones and none of them obtained their rice or oil supply from the aforesaid shop. Two of these unaffected families lived in the same house with an affected family but messed separately. In another instance, a group of four families was living side by side, one of which

getting its oil supply from the said shop was affected, whereas three others getting their oil from other sources remained unaffected although the members of these families had intimate contact. About 3 lb. of oil (sample No. 37) were collected from an affected family during this investigation, the epidemiological history associated with the oil being as follows :—

A family of seven bought as usual four seers of oil from K. M. C. and after one day's use left for Calcutta to join a social gathering. On their return home after a week they resumed the use of the oil. After eight days the first case developed and a few days later, three more cases occurred. Suspecting this supply of oil they abandoned its use. On making inquiries from the relatives who had joined the festivities in Calcutta they found that no one else had suffered.

(7) *Suri outbreak* (period of investigation—9th July, 1938 to 11th July, 1938).—Twenty cases were said to have occurred involving nine families residing in houses scattered about in one part of the town. At the time of investigation, all but three cases had recovered but a history suggestive of epidemic dropsy was obtained from all of them. These three uncured patients belonged to the family from which about 1½ lb. of oil (sample No. 39) was recovered. The epidemiological association of the oil with the incidence of the disease is as follows :—

Ten seers of oil were purchased from shop 'p' on the 16th January, 1938, about half of which was consumed next day in connection with certain celebrations. The use of the remaining oil was continued for some time when it was set aside on the advice of a local doctor. The first case appeared about eight days after the oil had been in use and it was not given up till four more cases had developed, the total number of persons in the family being eight. The only source of supply of six other affected families was also shop 'p', the two remaining families obtained their oil supply from other shops including shop 'p'.

(8) *The Calcutta Medical School outbreak* (period of investigation—9th June, 1938 to 14th June, 1938).—The resident members of the Institution can be classed as follows :—

Hospital patients	about 155
Nurses	22
House Surgeons	9
Resident Medical Officer	1
Clinical Pathologist	1
Cooks	6
Clerk and compounders	3
TOTAL	197

For purposes of messing they were distributed into four groups as follows :—

    I.   Patients (155).

    II. Nurses, the Clinical Pathologist, Cooks, Clerk and Compounders (32).

    III. House Surgeons (9).

    IV. Resident Medical Officer (1).

The outbreak.—Altogether 13 definite and five doubtful cases of epidemic dropsy occurred amongst the 32 members of group II. The other groups completely escaped. The distribution of the cases amongst members of group II was as follows :—

Group II.	Definite cases.	Doubtful cases.
Nurses	9	..
Clinical pathologist ..	1	..
Cooks	2	4
Compounder ..	1	1
Totals ..	13	5

The fact that the disease was confined to one mess, the members of which were engaged in different duties is striking. Further investigations brought out other points of interest in connection with the mustard oil theory. While the supply of foodstuff for groups III and IV was from different sources, the provisions for groups I and II were purchased, on behalf of the hospital authorities, from certain suppliers and the food served to the staff and the patients was the same with regard to the nature and quality of the food material, except for a few additional items, such as eggs, which were occasionally added for group II. The difference in the food of the two messes lay in the preparation of curries, considerably more spices and oil being used for group II than for group I. It was not possible to estimate the actual amount of oil used in the two messes. From the look of the curries and statement of the diet clerk and certain members of the staff, a fair estimate of the amount of oil used for group II would be four times that used for the patients. Taking into consideration further facts, e.g., that the amount of curry served to patients was much less than that served to the members of the staff, and the majority of the patients was given only one meal with curry a day, would lead to the conclusion that the amount of oil consumed by the patients was very little compared to that taken by the members of the staff and even amongst this group the nurses took less oil than the male members as most of them obtained only one meal from the mess. It would thus appear that the distribution of cases amongst those partaking of the same provisions was roughly proportionate to the amount of oil consumed. Unfortunately, only a few ounces of the suspected oil (sample No. 33) were available at the time of investigation.

Feeding experiments.

The main object of these experiments was to test the toxicity of the suspicious samples of oil obtained from the field with a view to carrying out suitable chemical and physical investigations. The experiments were conducted with oil obtained from (1) Khargpur, (2) Rangpur, and (3) Alamdanga, using jail-produced oil as

control. The general scheme was the same as described under experiment III in a previous communication (Lal and Roy, 1937*b*). However, the feeding was stopped as soon as the symptoms appeared and the object of the experiment was served. The results obtained are shown in Table II :—

TABLE II.

Feeding experiments on human volunteers with Khargpur, Rangpur, and Alamdanga oils.*

Serial number.	Sample number.	Source of oil.	Number of volunteers fed.	Number of definite cases developed.
1	19	Alamdanga	3	1
2	20	Rangpur	3	2
3	3	Khargpur	3	*Nil*
4	22	Jail-produced oil	3	*Nil*

Clinical details are given in the *Appendix*.
*For a discussion on the clinical significance of experimental cases a reference may be made to the *Cal. Med. Jour.*, **34**, No. 3, pp. 169–182.

DISCUSSION.

The eight outbreaks reported here present facts which can be explained on the basis of the theory put forward in a previous communication (Lal and Roy, 1937*b*), namely, that epidemic dropsy is caused by some deleterious substances (not a living virus) of unknown nature and origin contained in certain consignments of mustard oil. These reports, therefore, lend further support to this theory.

SUMMARY.

1. Eight outbreaks of epidemic dropsy are briefly reported.

2. The implications of the mustard oil theory have been tested in connection with these outbreaks.

3. Certain supplies of mustard oil incriminated on epidemiological grounds have been collected and their toxicity tested on human volunteers.

CONCLUSIONS.

As a result of further field observations and feeding experiments on human volunteers confirmation of the validity of the mustard oil theory of the ætiology of epidemic dropsy has been obtained.

ACKNOWLEDGMENTS.

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APPENDIX.

Examination of individual volunteers.

For the sake of brevity the negative findings are omitted and mention is made only of such signs and symptoms as have a direct bearing on the disease.

EXPERIMENTAL GROUP:—

No. 1 [I—'suspected' oil (sample No. 19) from Alamdanga]. Moderately-developed, active and bright Mohammedan male, 27 years, durrie weaver, been in the jail for 15 months, no previous illness, no complaint since in jail except slight enlargement of the right testicle—1 year back for few days after games. Height 5 ft. 7½ in.; weight 115 lb. Pulse 66. A few soft glands palpable in the inguinal regions.

Total amount of special food consumed:—

Dates: 22-2-38 to 11-3-38, missed 1 day as he refused the meal for want of appetite; rice: 229 ounces; oil: 29½ ounces.

Signs and symptoms after commencement of feeding:—

Subjective symptoms:—

3-3-38. Uneasiness and fullness of the stomach and loss of appetite after ingestion of 18⅙ ounces of oil.

4-3-38. Constipation and indigestion; wanted digestive medicines.

9-3-38. Loose stool.

17-3-38. Complains of œdema of legs after ingestion of 29¼ ounces of oil.

Physical signs:—

General appearance: dull and much reduced.

Weight: 21-2-38, 115 lb.; 5-3-38, 115 lb.; 12-3-38, 114 lb.; stools: 3-3-38, semi-solid, darkish; 7-3-38, semi-solid containing fair amount of mucus.

17-3-38. Slight œdema of both the legs with slight flush after the consumption of 29¼ ounces of oil.

Laboratory examinations:—

Blood: total R. B. C. count, 6-3-38, 5·38 million per c.mm.; 14-3-38, 4·90 million per c.mm.

Urine: nothing abnormal.

No. 2 (I—'suspected' oil from Alamdanga). Rather poorly-developed but active and bright Mohammedan male, 22 years, durrie weaver, been in jail for 2 months, no previous illness and no complaint since in jail. Height 5 ft. 3½ in.; weight 95 lb. Pulse 60.

A few small soft glands palpable in the inguinal region. Total amount of special food consumed:—

Dates: 22-2-38 to 11-3-38, missed a day for fever; rice: 123 ounces; atta: 87 ounces; oil: 26⅙ ounces. Signs and symptoms after commencement of feeding:—

Subjective symptoms:—

2-3-38. Loss of appetite after ingestion of 10⅓ ounces of oil.

4-3-38. Constipation.

5-3-38. Pain in the abdomen, loss of appetite, vomited twice last night.

26-3-38. Weakness and pain in the joints.

Physical signs:—

General appearance: dull and emaciated.

Weight: 21-3-38, 95 lb.; 5-3-38, 97 lb.; 12-3-38, 89 lb.

5-3-38. Fever (temperature 99·2°F.). Pulse 112.

Laboratory examinations:—

Blood: total R. B. C. count, 6-3-38, 3·7 million per c.mm.; 14-3-38, 4·22 million per c.mm.

Urine: nothing abnormal.

No. 3 (I—'suspected' oil from Alamdanga). Well-developed, active and bright Mohammedan male, 26 years. Coir-string preparer, been in jail for 8 months, no previous illness and no complaint

since in jail. Height 5 ft. 2½ in.; weight 98 lb. Pulse 56. A few soft small glands palpable in the inguinal region.

Total amount of special food consumed :—

Dates : 22-2-38 to 11-3-38, missed two days as he refused to take the meal due to diarrhœa : atta : 177 ounces ; oil : 23¼ ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms :—

4-3-38. Constipation after ingestion of 19 ounces of oil.

8-3-38. Loose motions, 6 or 7 times in a day and high coloured urine, feels rotten.

10-3-38. Feeling out of sorts and incomplete evacuation of bowels.

26-3-38. Complains of weakness and pain in the joints.

Physical signs :—

General appearance : dull and emaciated.

Weight : 21-2-38, 98 lb.; 5-3-38, 100 lb.; 12-3-38, 95 lb.

8-3-38. Loose stools : clay coloured and semi-solid in consistency.

Laboratory examinations :—

Blood : total R. B. C. count, 6-3-38, 4·44 million per c.mm.; 14-3-38, 4·63 million per c.mm.

Urine : nothing abnormal.

No. 4 (II—'suspected' oil sample No. 20 from Rangpur). Moderately-developed, active and bright Hindu male, 29 years, durrie weaver, been in jail for 2 years, no previous illness, no complaint since in jail except occasional pain in the epigastric region. Height 5 ft. 3½ in.; weight 110 lb. Pulse 62.

A few small soft glands palpable in the inguinal region.

Total amount of special food consumed :—

Dates : 22-2-38 to 11-3-38, rice : 182½ ounces ; oil : 23 ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms :—

24-2-38. Loose stools ; had 8 loose motions from last night after ingestion of 3½ ounces of oil.

1-3-38. Loss of appetite, loose motions with mucus in the stool.

3-3-38. Œdema of the legs after ingestion of 16⅓ ounces of oil.

4-3-38. Pain in the abdomen.

7-3-38. Fever last night.

8-3-38. Pain in the shoulder joint.

14-3-38. Weakness, blackening of face.

19-3-38. Tingling and formication.

24-3-38. Burning sensation all over the body and incomplete evacuation of bowels.

Physical signs :—

General appearance : dull, seedy, and much reduced.

Weight : 21-2-38, 110 lb.; 5-3-38, 111 lb.; 12-3-38, 106 lb.

11-3-38. Œdema of both the legs, pits on pressure after ingestion of 22⅔ ounces of oil.

14-3-38. Pigmentation of face and a flush on both the legs.

19-3-38. Anæmia, tongue coated, slight fever present, pulse 72, no abnormality of heart detected, knee-jerk normal (rather brisk), definite œdema of both legs and dorsum of feet with flush. Œdematous parts tender and painful.

Laboratory examinations :—

Blood : total R. B. C. count, 6-3-38, 4·8 million per c.mm.; 14-3-38, 3·74 million per c.mm.

Chemical examination of blood sera : 13-4-38, inorganic phosphorus 6·5 mg. per 100 c.c.

Urine : no abnormality found ; 17-3-38, urea concentration test normal.

No. 5 (II—'suspected' oil from Rangpur). Well-developed, active and bright, Hindu Punjabi male, 47 years, durrie weaver, been in jail for one month, no previous illness, no

complaint since in jail excepting a small hydrocele (right side). Height 5 ft. 5½ in.; weight, 126 lb. Pulse 60.

Total amount of special food consumed :—

Dates : 22–2–38 to 11–3–38, missed 3 days, refused the meal during that period, atta : 160 ounces; oil : 14⅛ ounces.

Signs and symptoms :—

26–2–38. Appetite diminished after ingestion of 70 ounces of atta and 6½ ounces of oil.
27–2–38. Irritating cough, vomiting last night.
28–2–38. Pain in the chest and right shoulder.
3–3–38. Complains of weakness.
4–3–38. Complains of constipation.
10–3–38. Complains of pain all over the body, small ulcer under the prepuce.
14–3–38. Œdema of both legs last evening after the ingestion of 160 ounces of atta and 14⅛ ounces of oil.
18–3–38. Slight blood during defæcation.
26–3–38. Cough, pain in abdomen and head.

Physical signs :—

Weight : 21–2–38, 126 lb.; 5–3–38, 127 lb.; 12–3–38, 121 lb.
11–3–38. Small ulcers under the prepuce.
14–3–38. Examined for œdema of legs and found to be suspicious.
18 -3–38. External piles.

Laboratory examinations :—

Blood : total R. B. C. count, 6–3–38, 4·73 million per c.mm.; 14–3–38, 4·61 million per c.mm.
Urine : no abnormality found.

No. 6 (II—'suspected' oil from Rangpur). Poorly-developed, dull-looking Hindu male, 37 years, coir pounder, been in the jail for a month, no previous illness, no complaint since in jail. Height 5 ft. 7 in.; weight 112 lb. Pulse 60. 1st sound in mitral area, slightly accentuated.

Total amount of special food consumed :—

Dates : 22–2–38 to 8–3–38 (refused co-operation after 8th March), rice : 162 ounces; oil : 20 ounces.

Signs and symptoms after the commencement of feeding :—

Subjective symptoms :—

26–2–38. Appetite slightly diminished after ingestion of 6½ ounces of oil.
2–3–38. Vomited last night and had 10 loose motions this morning. These symptoms continued up to 8–3–38. Later, complained of incomplete evacuation of bowels. Some times the stool was mixed up with mucus.
4–3–38. Pain in the hip.
9–3–38. Fever last night and was admitted to hospital. The fever was absent the next day but reappeared the day after.
10–3–38. Pain in the joints and burning sensation all over the body, the latter symptoms continued for a few days more.
24–3–38. Œdema of both the legs which increased in the evenings; extended up to the thigh after the ingestion of 20 ounces of oil.

Physical signs :—

Weight : 21–2–38, 112 lb.; 7–3–38, 112 lb.
9–3–38. Fever (temperature 100·4°F.). Pulse 80. Temperature subsided next day; 11–3–38, it rose up again up to 102°F. which continued for a few days more.
24–3–38. Definite œdema of both the legs, pits on pressure; a distinct flush on the œdematous parts.

Laboratory examinations :—

Blood : total R. B. C. count, 6–3–38, 2·52 million per c.mm.; 14–3–38, 2·06 million per c.mm.
Chemical examination of blood sera : 13–4–38, inorganic phosphorus 4·7 mg. per 100 c.c., uric acid 4·48 mg. per 100 c.c., and creatinine 1·25 mg. per 100 c.c.
Urine : 14–3–38, traces of albumin; some hyaline casts, epithelial cells and a few pus cells.

INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY.

Part VIII.

STUDIES ON THE NATURE AND ORIGIN OF CERTAIN (? TOXIC) SUBSTANCES PRESENT IN THE SUPPLIES OF MUSTARD OIL ASSOCIATED WITH OUTBREAKS OF EPIDEMIC DROPSY.

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IN a previous communication (Lal and Roy, 1937) it was stated that all the available evidence was suggestive of the presence of some toxic chemical substance rather than of a living virus in the mustard oil associated with outbreaks of epidemic dropsy. However, so far little was known of the nature and origin of the poisonous factor beyond the facts that it was heat-stabile and that there were reasonable grounds to believe that it was something other than allylisothiocyanate (Lal, Ahmad and Roy, 1938). Both for practical and academic reasons it was necessary to investigate these problems. Before, however, these studies could be undertaken it was necessary to secure supplies of proved toxic mustard oil in sufficient quantities. As a result of further field investigations followed by human feeding experiments (Lal and Roy, 1939) we were able to secure two such supplies one of which (Rangpur oil) proved more toxic than the other (Alamdanga oil).

This method of procuring samples of oil of known potency has obvious limitations. In the first place, for reasons already given (Lal, Ahmad and Roy, *loc. cit.*) opportunities for collecting large quantities of oil definitely associated with an outbreak of the disease are infrequent and in the second place facilities for conducting human feeding experiments to which recourse has to be made, are extremely limited. As a preliminary to further investigations, it was therefore necessary to devise simpler tests to differentiate between the safe and the unsafe oils. Apart from purposes of research, the development of these tests would be an important contribution towards the practical control of the disease because, given the differential tests, the health authorities should be in a position to suppress the sale of such supplies as were likely to endanger public health.

DIFFERENTIAL TESTS.

The problem was approached along three different lines, viz., (*a*) biological, (*b*) chemical, and (*c*) physical.

A. BIOLOGICAL TESTS.

The investigations were conducted by three different methods, viz.,

> (*i*) feeding experiments on animals, more or less on the lines followed in experiments on human subjects,
>
> (*ii*) subcutaneous injections in monkeys, and
>
> (*iii*) prolonged application of the oil to the scarified skin of man.

(*i*) *Feeding experiments on animals.*—Experiments were performed on a series of animals including rats, cats, guinea-pigs, pigeons, and pigs. On account of certain developments reported hereinafter the scope of these experiments was considerably extended and the results cannot be conveniently set out here. We propose to deal with this aspect of the problem in another communication.

(*ii*) *Subcutaneous injections in monkeys.*—Two young male monkeys (*S. rhesus*) were selected. One received bi-weekly subcutaneous injections of 1 c.c. of heated Rangpur oil for five weeks and the other received similar injections of heated jail-produced oil. Later, the amount of oil was increased to 1½ c.c. and the injections were given on alternate days for a week. In the following week daily injections of 2 c.c. of the respective oils were given. The total amount of oil received by each animal in course of 54 days was 28 c.c. Both animals increased slightly in weight, the one on jail-produced oil showing a little more increase than the other. In both cases hard nodules developed under the skin at the site of injections which slowly disappeared. No other local or general effects were observed.

(*iii*) *Experiments on human skin.*—The skin of epidemic-dropsy patients exhibits definite clinical and pathological features which are considered pathognomonic of the disease. This suggested the possibility of producing specific local lesions by prolonged application of the potent oil to scarified skin. The technique of the experiment was as follows :—

A small patch of skin, of the size of a two-anna piece, on the dorsum of either arm, was gently scarified with a rotary vaccinator without drawing any blood.

On the right side, a pad of lint soaked in the heated Rangpur oil was placed, and on the other was placed a similar pad soaked in heated jail-produced oil. The pads were kept in position continuously (except for the time of bathing) by means of vaccination shields and elastic bands. The pads were changed daily and the scarification was performed twice a week. The experiment was continued for a month. Three subjects were treated in this manner. Observations made with a hand lens failed to demonstrate any local reaction.

B. Chemical Tests.

In the problem of developing chemical tests we were up against a blind wall. Nothing was known about the nature of the toxic substance except that it was heat-stabile and soluble in oil. There were no leads to follow. As on previous occasions, the potent oil was found to satisfy the chemical standards as laid down under the rules made by the Local Government under the Bengal Food Adulteration Act of 1919.

Further tests were carried out to detect other possible adulterants such as cotton-seed oil, sesame oil, almond oil, tung oil, archis oil, liver oil, and other drying and non-drying vegetable oils. Besides, a large number of other chemical reagents were used. The toxic oils showed no distinctive reactions except with Crace Calvert's and Cavalli's reagents. On shaking a little oil with either of these reagents and letting the mixture stand for a little while, the upper oily layer separated from the watery layer and the latter developed an orange-red colour in the case of the toxic oil but remained colourless in the case of jail-produced oil. The coloration was deeper with Rangpur oil than with Alamdanga oil. It was recognized that in both these tests, nitric acid was a common reagent. Hauchecorn's test was, therefore, performed. This consisted of vigorous shaking of a little oil with an equal volume of concentrated nitric acid. As before, in the toxic oil but not in the control, a brownish red colour developed in the bottom acid layer, thus suggesting a differential reaction. To establish the value of this and other tests presently to be described, a large number of samples of mustard oil with varying degrees of association with the disease were employed.

Cupric acetate test.

Another chemical test was devised at the suggestion of Dr. S. S. Bhatnagar. This consisted of heating in a water-bath, for about 15 minutes, a mixture of the oil acidified with glacial acetic acid and three per cent solution of cupric acetate. With the toxic oil a precipitate was formed and the colour of the watery layer changed from blue to green but this change did not occur with a non-toxic oil. This reaction, therefore, suggested another differential test.

C. Physical Tests.

(*i*) *Spectrophotometry.*—One per cent alcoholic solution of the toxic and the non-toxic oils were subjected to spectrophotometric studies. The instrument used was a Quartz Spectrophotograph by Carl Zeiss. It had a dispersion of about five inches with a range from 3,500 A. U. to 2.000 A. U. Tungsten spark or hydrogen discharge tubes were used as sources of light. The solution under investigation

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was placed in a Baly's tube and absorption spectrophotographs were taken. The results are shown in Plate X, figs. 1, 2, and 3. It will be observed that the general nature of the spectrum is the same in the three cases but the toxic oils show a broad absorption band between 2,900 A. U. and 2,600 A. U. with the maxima near about 2,750 A. U. The degree of absorption is greater in the case of Rangpur oil than in Alamdanga oil, the extinction co-efficient (at 2,750 A. U.) being 1·2 for the former and 0·6 for the latter.*

(*ii*) *Fluorescence test.*—When the toxic oil was exposed to ultra-violet radiation it exhibited a characteristic bluish violet fluorescence. The intensity of fluorescence was greater in the Rangpur oil than in the Alamdanga oil. It was absent in the jail-produced oil. A careful observer may, in fact, observe definite fluorescence in the highly toxic oil in direct sunlight.

We thus have two physical and two chemical reactions given by the toxic oils and not by the control oil, which suggest bases for differential tests. Since, as stated above, differential tests are of great practical importance in the prevention and control of the disease and since they are likely to materially help in further investigations, it is of paramount importance to establish the validity of their differential character. It is, therefore, necessary to apply the tests suggested by the differential reactions of toxic and control oils to a large number of known poisonous and non-poisonous samples of mustard oil. The difficulties of obtaining sufficiently large number of samples of poisonous oils and proving their toxicity by human experiments have already been referred to. It was, therefore, decided to collect samples of mustard oil epidemiologically associated with the disease. This was by no means an easy task. Attention had been drawn, on previous occasions, to the difficulties in obtaining samples of oil which the victims had consumed prior to acquiring the disease. However, our field staff and health officials, mostly Sanitary Inspectors in various parts of Bengal, were able to collect 282 samples of mustard oil together with the relevant epidemiological histories in each case. A standard form was used for collecting epidemiological information (vide *Appendix I*). In most cases, the forms were properly completed but in some cases, they were either incompletely filled or contained obvious defects. For these reasons 74 samples had to be rejected. Besides, a number of these samples were collected from shops which had supplied oil to the affected families or from the affected families from supplies in use at the time of the collector's visit, which in many cases occurred long after the appearance of the first case in the family. In such instances the association of the particular sample of oil with the disease could not, of course, be established. On the other hand, the epidemiological evidence in some cases was sufficiently strong to incriminate certain samples, and from the proportion of cases to the number of people in the family a rough estimate of the toxicity of the oil could be made. Our previous experience with experimental subjects (Lal and Roy, 1937), provided a basis for estimating the average amount and period of consumption of toxic oil required to produce the symptoms. We were thus able to classify the samples of oil in different grades according to their epidemiological association and toxicity (*vide* Table I). Further details regarding the

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* Extinction co-efficient $E = \dfrac{1}{d}$, where d = length in centimetres of the solution required to produce complete absorption.

criteria for classification are given in *Appendix II.* The samples of known non-toxic oils used as controls were obtained from supplies which had been in use for sometime in the jails or in private families without causing ill effects. For sources of some of these samples, reference may be made to a previous communication (Lal, Ahmad and Roy, *loc. cit.*). Having assessed the epidemiological association, each sample of oil was subjected to the nitric acid, cupric acetate and fluorescence tests in the manner already described. From Table I, which summarizes the results obtained in different grades of oil, it will be seen that the cupric acetate test has proved-differential in 21 out of 23 incriminated oils and in all the 23 safe oils. The same results have been obtained with nitric acid test except that it has given positive results with two samples having epidemiologically negative histories. The fluorescence test is positive in 22 out of the 23 samples of oil having positive epidemiological histories but it is also positive in 11 out of 23 samples of oil not associated with the disease. Unfortunately in a large majority of the samples of class III the epidemiological history is not so definite. However, by careful scrutiny of the available data in each case the samples in the class can be divided into two groups, viz., those in which association with the disease may be presumed and those in which such presumption is not justified. Here again, there is practically a complete correspondence between the results of the two chemical tests and there is suggestive evidence of their differential character. The fluorescence test is positive in a larger proportion of samples than the chemical tests both in the suggestive and the non-suggestive groups. From a detailed consideration of the individual samples, it was found that the samples giving positive reaction with cupric acetate also gave positive nitric acid and fluorescence tests and that the samples giving positive nitric acid test but negative cupric acetate test gave positive fluorescence test.

TABLE I.

Results of physical and chemical tests in various epidemiological classes of samples of oil.

Test.		Epidemiological history and toxicity positive (class I).	Epidemiological history and toxicity negative (class II).	EPIDEMIOLOGICAL HISTORY AND TOXICITY INDEFINITE.	
				Suggestive (class IIIa).	Not suggestive (class IIIb).
Nitric acid test ..	Positive	21	2	37	42
	Negative	2	21	15	68
Cupric acetate test	Positive	21	0	36	39
	Negative	2	23	16	71
Fluorescence test	Positive	22	11	47	77
	Negative	1	12	5	33

`Tables II and III set out the results obtained with individual samples in classes I and II. The extinction co-efficients of certain samples are also shown in these tables. Although an attempt has been made to estimate the degree of toxicity and intensity of reactions in each case, it is not intended to claim any accuracy with regard to quantitative measurements except in the case of extinction co-efficients. It will be observed that the results of all the tests fit in well with the results of human experiments in respect of samples from Rangpur and Alamdanga oils but, as the data now stand, an exact correspondence between the degree of toxicity and the intensities of different reactions is lacking. In general it may be stated that of all the tests, the fluorescence test appears to be the most sensitive and the spectrophotographic test the least so. This hypothesis might explain the presence of a slight degree of fluorescence in known non-poisonous oils. The result of spectrophotographic analysis is anomalous in the case of sample No. 52 for which no explanation is forthcoming. The results of samples Nos. 3 and 13 in Table III and the relative intensity of reactions shown in Table II would suggest that the nitric acid test is the more sensitive of the two chemical tests.

TABLE II.

Differential tests on samples of oil associated with outbreaks of the disease.

Serial number.	Sample number.	Toxicity.	Nitric acid test.	Cupric acetate test.	Fluorescence test.	Extinction co-efficient at 2,750 A. U.	REMARKS.
1	15	+++	++	+	++++	..	
2	20	+++	+++	+++	++++	1·2	Rangpur oil.
3	294	+++	+	+	+++	..	
4	297	+++	+	+	+++	..	
5	308	+++	++	++	+++	..	
6	37	+++	+	++	++	0·7	
7	39	+++	+++	+++	++	1·0	
8	118	+++	++	+	++	..	

TABLE II—*concld.*

Serial number.	Sample number.	Toxicity.	Nitric acid test.	Cupric acetate test.	Fluorescence test.	Extinction co-efficient at 2,750 A. U.	REMARKS.
9	132	+++	+++	+	++	0·6	
10	152	+++	+++	+	++	0·8	
11	167	+++	+	+	++	··	
12	301*	+++	—	—	—	··	
13	288	++	+	+	+++	··	
14	97	++	+	+	++	··	
15	135	++	++	+	++	0·5	
16	299	++	+	+	++	··	
17	309	++	++	++	++	··	
18	33†	++	++	++	+	··	
19	69*	++	—	—	+	··	
20	19	+	++	+	+++	0·6	Alamdanga oil.
21	96	+	+++	++	+++	0·6	
22	269	+	+	+	+++	··	
23	94	+	++	++	+++	··	

* These samples were submitted by health officials. They apparently satisfied the conditions required to associate the oil with the disease. Since they were obtained from houses of educated people, the information may be presumed to be reliable. However, it is not known who actually collected the samples, labelled and despatched them.

† Fluorescence test performed on acid extract as quantity was too small for testing by the usual method.

TABLE III.

Differential tests on samples of oil not associated with outbreak of the disease.

Serial number.	Sample number.	Nitric acid test.	Cupric acetate test.	Fluorescence test.	Extinction co-efficient at 2,750 A. U.	REMARKS.
1	14	—	—	—	..	
2	21	—	—	—	..	Jail-produced oil.
3	22	—	—	—	..	Another consignment of jail-produced oil.
4	43 (1)*	—	—	+	0·0	
5	45 (3)	—	—	—	0·4	
6	46 (4)	—	—	+	0·0	
7	47 (5)	—	—	+	0·5	
8	48 (6)	—	—	+	0·0	
9	50 (8)	—	—	—	0·6	
10	51 (9)	—	—	++	0·4	
11	52 (10)	—	—	+	0·8	
12	53 (11)	—	—	+	0·6	
13	54 (12)	—	—	+	0·4	
14	55 (13)	—	—	—	0·4	
15	56 (15)	—	—	—	0·5	
16	57 (16)	—	—	+	..	
17	155	—	—	—	..	
18	220	—	—	—	..	
19	221	—	—	—	..	
20	298	—	—	—	..	
21	40	—	—	—	..	
22	3	+	—	+	..	
23	13	+	—	+	..	

* The numbers in brackets correspond with the sample numbers in Table X given in a previous communication (Lal, Ahmad and Roy, *loc. cit.*) to which reference may be made for information regarding their sources and histories.

The studies detailed above would appear to establish, in a general way, the differential nature of these tests. It is, therefore, recommended that they should be used for purposes of directing preventive and control measures against the disease. Since the two chemical tests require little or no equipment and can be easily performed by health officials in the field they should be extensively used. In this respect they mark an important advance in our knowledge of the disease and its prevention and thereby equip the health officials, and the intelligent consumer with an effective weapon to guard against the disease. It should, however, be clearly understood that it is premature to use the tests, in the present state of their development, as bases for action involving legal complications. Further studies mentioned in a later section of this communication suggest that most of the supplies of mustard oil produced in this country are liable to contain some amount of the toxic substance but the harmful effects are produced through its cumulative action, only when the concentration of the poison exceeds certain unknown limits. It is, therefore, necessary to establish certain definite standards about the permissible amount of the toxic substance in the oil and to work out if possible the correspondence of these standards with the intensities of the differential reactions.

SOURCE OF THE TOXIC SUBSTANCE.

The preventive measures can be more effectually applied and loss to producers of oil can be prevented if the source of the toxic substance can be discovered. As previously mentioned, there are three possibilities, viz., (*i*) the deterioration of mustard seed due to infection with fungoid or other forms of organisms through faulty storage or otherwise, (*ii*) adulteration of the oil with some cheap poisonous oil, and (*iii*) admixture of the mustard seed with some poisonous seeds either deliberately or inadvertently.

We have investigated these possibilities with the following results :—

(*i*) *Deteriorated mustard seeds.*—Two supplies of mustard seeds showing obvious signs of deterioration and emitting strong musty odour were procured. Oils expressed from these seeds were tested by means of feeding experiments on two groups of three volunteers each. The general scheme of the experiments was the same as detailed in experiment III reported previously (Lal and Roy, 1937). One experiment lasted for 19 days and the other for 20 days. The average amount of oil consumed per individual was 39 and 45 ounces respectively. No ill effects were observed in any of the experimental subjects. It may perhaps be of interest to note here that from the Rangpur oil Dr. R. K. Goyal was able to grow a fungus which was reported by Professor S. R. Bose as *Mucor* Sp. commonly found in fermenting rice.

(*ii*) *Adulteration of mustard oil with some other oil.* This subject has already been discussed.

(*iii*) *Adulteration of the mustard seeds with other seeds.*—Since the millowners and dealers of the mustard oil have frequently been found to be the first victims of the disease in an outbreak, the indications are that they are not responsible for the adulteration of the oil or seeds with

any known poisonous substance and are ignorant of such adulteration, if any.

The mustard seed stocked in the oil mills for crushing are rarely, if ever, found without admixture with some variety of extraneous seeds. For reasons already stated it was not an easy task to obtain mustard oil which had been consumed by the victims prior to the onset of the disease. On account of these and other reasons it was not found possible to secure samples of seeds from which toxic oil might have been expressed. No extraneous seeds, therefore, could be incriminated in this way. However, in the course of a discussion on the implications of the mustard oil theory and particularly on the report of an outbreak by Kamath (1928) in which Dr. R. K. Goyal took part, a profitable line of approach was suggested. Reference to this publication was made in a previous communication (Lal, 1938) in connection with the general discussion on the ætiological rôle of mustard oil in epidemic dropsy. It was pointed out that besides Kamath's work we knew of no published evidence in support of the criticism that outbreaks of the disease may occur amongst non-consumers of mustard oil. On referring back to this report it was evident that the outbreak described therein was similar in all respects to what we were accustomed to find in our field investigations and although no definite statement was made to exclude the use of mustard oil amongst the victims, the text suggested that gingelly oil was in common use and that oil, expressed from a wild poisonous plant locally known as 'odissimari', was often employed as an adulterant. The plant and seeds of 'odissimari' were obtained from Orissa through the courtesy of Lieut.-Colonel G. Verghese, I.M.S., which Professor S. P. Agharkar kindly identified for us as *Argemone mexicana*. The differential tests mentioned in the previous section were applied to oil obtained from seeds of this plant mixed in various proportions with pure mustard oil. It was found that a mixture containing about 6 per cent of *Argemone* oil gave the same reactions as Rangpur oil (*vide* Table IV and Plate XI, figs. 4, 5, and 6).

TABLE IV.

Results of differential tests on Argemone *oil mixed with mustard oil in different proportions.*

Sample number.	Nature of oil.	Nitric acid test.	Cupric acetate test.	Fluorescence test.	Extinction co-efficient (at 2,750 A. U.).
183	Pure *Argemone* oil ..	Acid layer crimson coloured.	Very strongly positive.	Highly fluorescent.	5·0
184	Jail-produced oil containing 10 per cent of *Argemone* oil.	++++	++++	++++	1·0
185	Jail-produced oil containing 5 per cent of *Argemone* oil.	++	++	++++	0·8

PLATE X.

Source.—Hydrogen discharge.

−2,500 −3,000 −3,500 −4,000

5 · 0
2 · 5
1 · 6
1 · 2
1 · 0
0 · 8
0 · 7
0 · 6
0 · 55
0 · 5
0 · 45
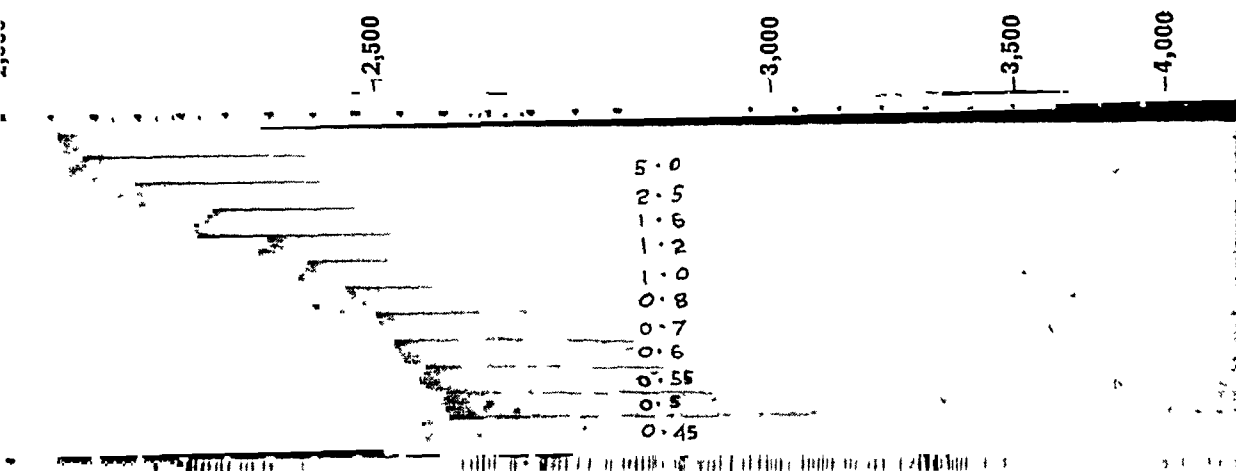
FIG. 1.   Jail-produced oil (No. 22).

5 · 0
2 · 5
1   6
1 · 2
1   0
0 · 8
0 · 7
0 · 6
0   55
0 · 5
0 · 45

FIG. 2.   Alamdanga oil (No. 19).

5   0
2 · 5
1 · 6
1 · 2
1 · 0
0 · 8
0 · 7
0 · 6
0 · 55
0 · 5
0   45
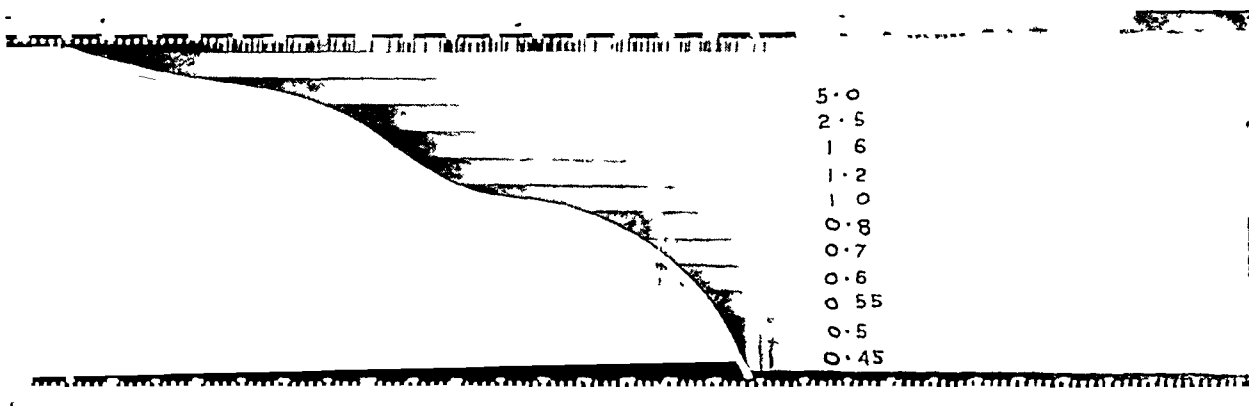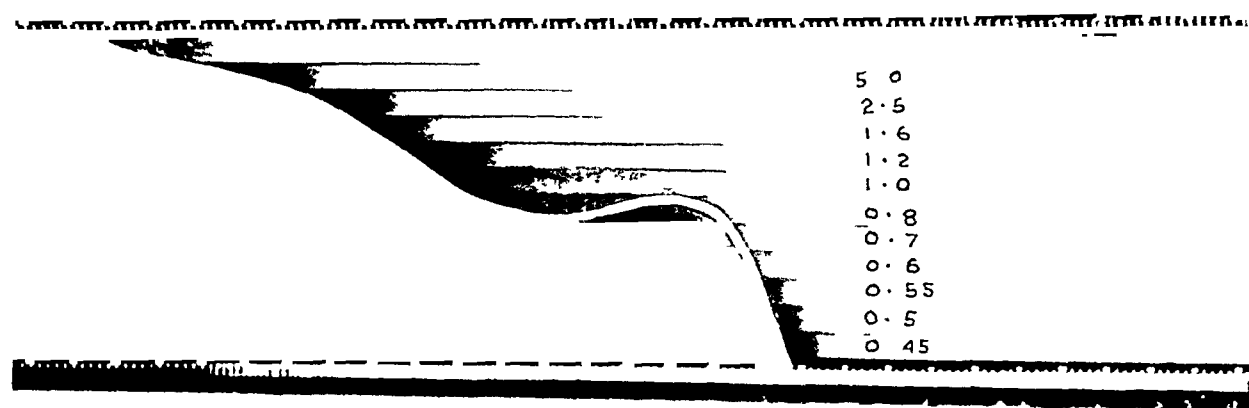
FIG. 3.   Rangpur oil (No. 20).

PLATE XI.

Source.—Tungsten steel spark.
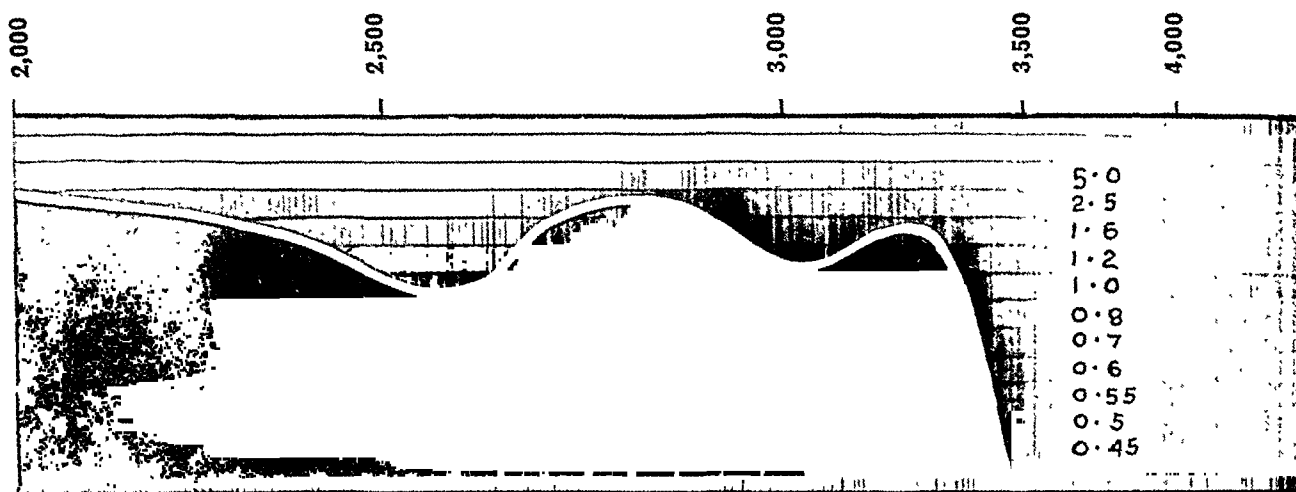
FIG. 4. Pure *Argemone* oil.
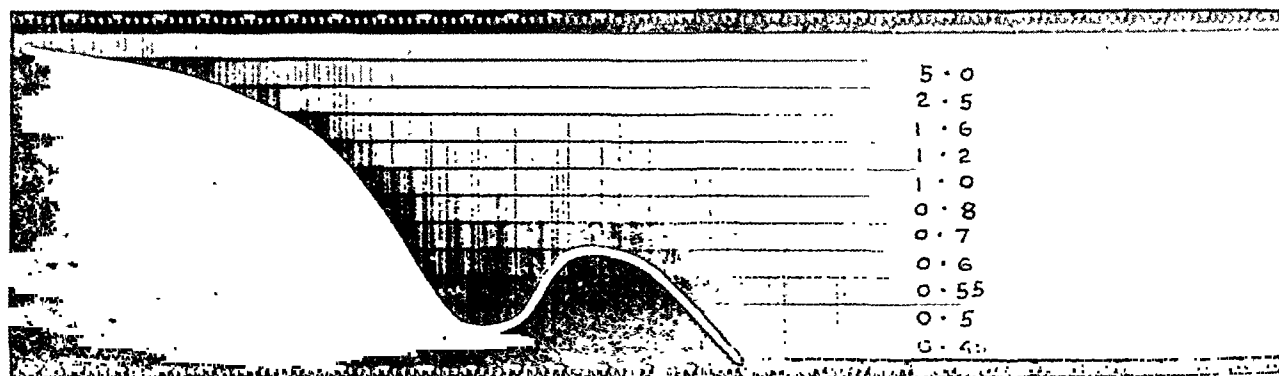
FIG. 5. Jail-produced oil containing 5 per cent *Argemone* oil.
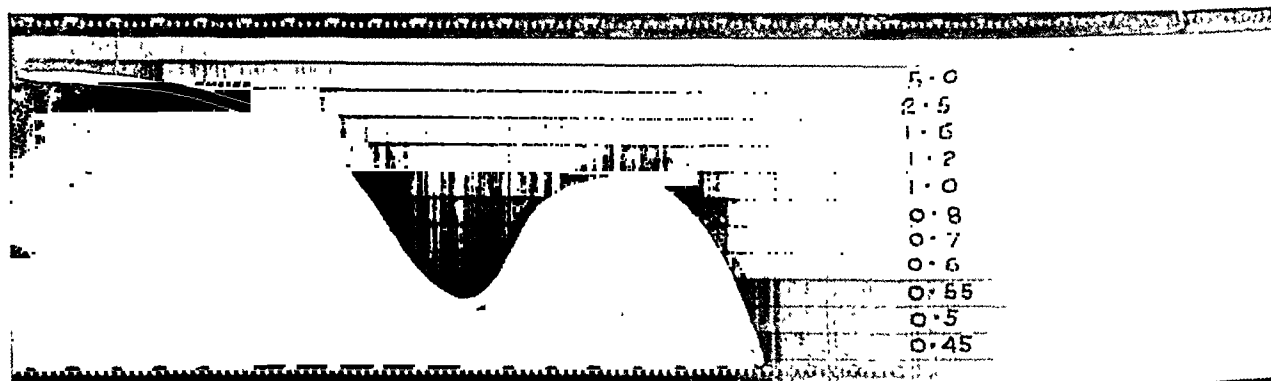
FIG. 6. Jail-produced oil containing 10 per cent *Argemone* oil.
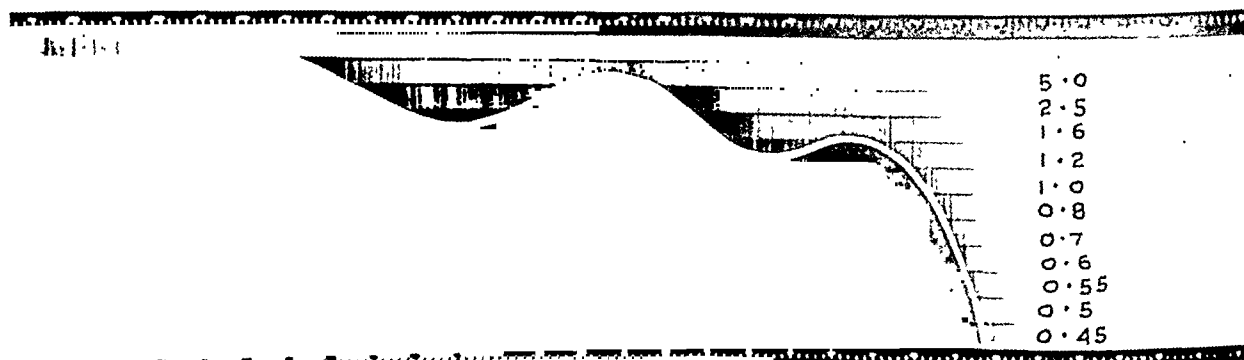
FIG. 7. U. V. absorption spectrum of white crystalline substance in alcoholic solution.

The fact that *Argemone* seeds impart the physical and chemical properties of toxic oil to mustard oil was demonstrated by a simple experiment. Oil was expressed from a sample of mustard seeds containing about 10 per cent of *Argemone* seeds. On applying the differential tests it gave positive reactions. The *Argemone* seeds were then carefully removed from the mixture and oil was expressed. It gave entirely negative results with the tests.

The question naturally arises whether *Argemone* seeds are in fact found mixed with mustard seeds. Stocks of mustard seeds waiting to be crushed in 40 different mills in and around Calcutta were examined with the following results :—

1. Number of samples in which no *Argemone* seeds could be seen = 23.
2.   ,,   ,,   ,,   ,,   ,,   ½ to 1 per cent of *Argemone* seeds were found = 12.
3.   ,,   ,,   ,,   ,,   ,,   1 to 5   ,,   ,, *Argemone* seeds were found = 5.
4.   ,,   ,,   ,,   ,,   ,,   over 5   ,,   ,, *Argemone* seeds were found = 0.

These examinations were made at a time when epidemic dropsy was not widely prevalent in Calcutta and no serious epidemics occurred in the following three or four months.

It may, therefore, be provisionally concluded that seeds of *Argemone mexicana* which bear a superficial resemblance to the black variety of mustard seeds (*Brassica napus*) find their way in stocks of mustard seeds through accidental or wilful adulteration by the suppliers, without the knowledge of the millowners and that the oil expressed from mustard seeds containing sufficient quantities of those seeds is responsible for the outbreaks of the disease. In this connection an old report on ' katakar ' oil poisoning (Sarkar, 1926) is of interest, ' katakar ' being a vernacular name for *Argemone mexicana*. The author relates the story of an outbreak of a disease resembling epidemic dropsy which involved all the 28 members of three families and of which he had received reports. It is stated that mustard oil was pressed in an oil mill which had been previously used for expressing ' katakar ' oil. The victims had consumed this oil two or three days prior to the onset of the symptoms. Our present findings lend support to Sarkar's conclusion that the disease was due to the consumption of mustard oil which had been accidentally adulterated with ' katakar ' oil and his report would have been of importance in unravelling the mystery of the ætiology of the disease but for the fact that his conclusion was based on fragmentary and otherwise unsatisfactory evidence obtained mostly by hearsay. As a matter of fact, our attention had previously been drawn to this report by the author but on account of serious discrepancies with regard to the date on which the oil was expressed and the date on which it was consumed it had failed to carry conviction. Even if it is conceded that ' Janmastami ', the festival on which the cakes fried in the adulterated oil are said to have been taken, occurred later than the date on which the oil was expressed, it appeared unlikely that consumption of food cooked in the oil on a single occasion should cause, within two or three days, severe symptoms and even mortality ; at least such a happening was contrary to our experience. However, we are not familiar with the symptoms and sequelæ of acute *Argemone* oil poisoning and therefore the interpretation of the statements contained in Sarkar's report may, at present, remain under doubt.

For a final proof of the hypothesis, appeal must necessarily be made to human experiments. Experiments in the usual way were conducted with jail-produced oil adulterated with 10 per cent oil of *Argemone mexicana*, on three subjects, but before a sufficient quantity of the mixture could be administered, the volunteers withdrew co-operation, and the experiment had to be abandoned.

NATURE OF THE TOXIC SUBSTANCE.

In the course of our search for a differential test we had occasion to test the reducing properties of the oils. The technique employed was to titrate the oil against $N/10$ potassium permanganate solution with and without the addition of 1 in 4 sulphuric acid. The mixtures were incubated at 37°C. for varying periods before titration. Both heated and unheated oils were used. The results are set out in Table V. Although the oxygen absorption values are not very regular, the results in general indicate that Rangpur oil contains the largest amount of reducing substance and the jail-produced oil the least. It may also be seen that the reducing substance, whatever its chemical constitution may be, is heat-stabile.

TABLE V.

Oxygen absorption capacity of samples of three test oils $(N/10 \ KMnO_4$ *solution, in c.c., absorbed per one g. of oil).*

Test oils.	JAIL OIL.		ALAMDANGA OIL.		RANGPUR OIL.	
	With acid.	Without acid.	With acid.	Without acid.	With acid.	Without acid.
(A) Three minutes' incubation at 37°C.						
(*a*) Unheated ..	1·07	1·37	1·03	1·5	1·53	2·8
(*b*) Heated at 100°C. ..	0·9	1·45	1·0	1·52	1·64	2·59
(B) Three hours' incubation at 37°C.						
(*a*) Unheated ..	9·4	6·4	9·2	..	10·8	..
(*b*) Heated at 100°C. ..	8·9	..	10·4	..	10·7	..
(C) Twenty-four hours' incubation at 37°C.						
(*a*) Unheated ..	9·57	7·1	9·85	7·4	10·8	8·5
(*b*) Heated at 100°C. ..	9·0	7·5	8·5	8·2	10·4	8·9

Since organic toxins are likely to contain nitrogen, the nitrogen content of the toxic and control samples of oil was investigated. Kjeldahl's method was employed. The results are shown in Table VI. It will be seen that the amount of nitrogen

is largest in Rangpur oil and least in jail-produced oil, Alamdanga oil occupying an intermediate position. Since, however, it was found that Rangpur oil contained 3·51 mg. of allylisothiocyanate per g. of oil, Alamdanga oil 2·82 mg. per g. and jail-produced oil 2·57 mg. per g., it was considered necessary to eliminate this source of difference in the nitrogen content. Samples of oil to be tested were freed from allylisothiocyanate by the technique employed on previous occasions (Lal, Ahmad and Roy, *loc. cit.*). The results are shown in Table VI. It will be seen that the nitrogen content of Rangpur and Alamdanga oils, apart from that contributed by allylisothiocyanate or other volatile substances, is much higher than that of the jail-produced oil.

TABLE VI.

Estimation of total nitrogen by Kjeldahl method (mg. per 100 g. of oil).

Samples.	Total nitrogen mg. per 100 g. of oil.
(*a*) UNHEATED :	
(*i*) Jail oil	35·6
(*ii*) Alamdanga oil	48·2
(*iii*) Rangpur oil	50·3
(*b*) SAMPLES FREED FROM ALLYL-ISOTHIOCYANATE :	
(*i*) Jail oil	10·6
(*ii*) Alamdanga oil	18·3
(*iii*) Rangpur oil	20·8

With a view to isolating the toxic substance, Rangpur oil and oil of *Argemone mexicana* were split into the saponifiable and non-saponifiable fractions in the usual way. A white crystalline substance soluble in hot alcohol and dilute hydrochloric acid was obtained. It melted at 185°C. and left no ash on incineration. It gave intense reactions on applying the differential tests. The spectrophotograph is shown in Plate XI, fig. 7. The crystals were obtained in fairly large amounts from *Argemone oil*, but in smaller quantities from Rangpur oil. By means of acid extraction, a minute trace of this substance could be isolated from jail-produced oil, but not from oil expressed out of mustard seeds from which seeds of *Argemone mexicana* had been carefully removed. Besides the white crystalline substance, certain other compounds have also been isolated from the oil of *Argemone mexicana* and from the potent oil, but these substances have failed to give the differential tests.

These results indicate that the white crystalline substance, mentioned above, is responsible for the differential tests and that it is derived from seeds of *Argemone mexicana*. It is, however, quite another matter as to whether this substance is the

factor to which toxic effects are due.   As a matter of fact, a feeding experiment on human volunteers with the non-saponifiable fraction obtained from Rangpur oil and added to an equivalent amount of jail-produced oil has failed to prove an ætiological rôle of this fraction (for details see *Appendix III*).   This observation may suggest that the white crystalline substance is not likely to be the toxic element.   However it is possible that other factors are involved.   For example, it is possible that the chemical substance might have been greatly reduced in amount or modified in the course of preparation of the non-saponifiable fraction.

Summary.

1.   The scope of conducting human feeding experiments to distinguish between poisonous and non-poisonous oils being limited, possibilities of developing simpler differential tests have been investigated along three lines, namely, biological, chemical, and physical.

2.   Two chemical tests, namely, nitric acid and cupric acetate tests, and two physical tests, namely, fluorescence and spectrophotometric tests, have been described.

3.   The chemical and the fluorescence tests have been applied to 208 samples of mustard oil, variously associated with epidemic dropsy and obtained from different parts of Bengal.   The results indicate that these tests can distinguish between samples of mustard oil containing the toxic substance and those free from it.   The spectrophotometric studies have been conducted with nine samples associated with the disease and 12 samples not so associated.   The scope of this test appears to be limited.

4.   Evidence is presented to support the hypothesis that the seeds of *Argemone mexicana*, bearing a superficial resemblance to the black variety of mustard seeds (*Brassica napus*), find their way into stocks of mustard seeds through accidental or wilful adulteration by the suppliers and that oil expressed from mustard seeds containing sufficient quantities of seeds of *Argemone mexicana* is responsible for outbreaks of the disease.

5.   A white crystalline substance has been isolated from oil of *Argemone mexicana* and from a proved toxic oil but not from pure mustard oil.   This substance has been found to be responsible for the differential tests.   Preliminary studies suggest that the toxic symptoms may not be due to this substance in the form in which it is known to us.   However, further studies are indicated before a definite conclusion can be arrived at.

Conclusions.

1.   One physical and two chemical tests are now available for routine examination of mustard oil to differentiate between supplies containing a toxic substance responsible for epidemic dropsy and those free from it.   It is recommended that these tests be applied by health officials for purposes of prevention and control of the disease.

2.   It is provisionally concluded that the toxic substance contained in the mustard oil which is responsible for the disease, is derived from seeds of *Argemone*

mexicana, which are frequently found in stocks of mustard seeds in oil mills and which may at times be present in considerable quantities.

3. A white crystalline organic compound which has been isolated from oil of *Argemone mexicana* and from a toxic oil is responsible for the differential tests, but the rôle of this substance in producing symptoms of the disease has not yet been determined.

·ACKNOWLEDGMENTS.

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APPENDIX I.

Form for particulars about the samples of mustard oil.

Serial number of samples.	Date of collection and quantity collected.	Collected from affected or unaffected family or from shop.	If from family, date of purchase of sample by the family.	Quantity purchased of which the sample is a part.	Monthly consumption by the family (amount).	Date of onset of 1st case.	Total persons in the family with the ages.	Total cases in the family with their ages.	REMARKS.

APPENDIX II.

CLASS I.

Epidemiology and toxicity positive.

One or other of these samples was actually consumed by one or more affected families for two weeks or more before the appearance of the first case in the family. In some cases, however, the samples were consumed only for about a week but there was evidence to suggest that oil from the same source, possibly from the same consignment, was in use for some time previously.

CLASS II.

Epidemiology and toxicity negative.

Most of these samples were collected from families in which no cases of epidemic dropsy had occurred after consuming the sample for two weeks or more. The remaining few were collected from a local jail or were expressed by us from mustard seeds and had been proved negative by feeding experiments on human volunteers.

CLASS III.

Epidemiology and toxicity indefinite.

(a) *Suggestive :—*

These samples were bought a week or less before the appearance of the first case in the family or belonged to some brands of oil (whether from shop or family) which were, at the same period, suspected by many sanitary inspectors independently and in widely separated areas. When collected from families, only those samples were put in this class as were associated with multiple cases.

(b) *Not suggestive :—*

This class consisted of those samples which could not be put in any of the previous classes on the basis of the information supplied.

APPENDIX III.

Examination of individual volunteers.

For the sake of brevity the negative findings are omitted and mention is made only of such signs and symptoms as have a direct bearing on the disease.

A. EXPERIMENTAL GROUP :—

No. 7 (III—'non-saponifiable' fraction of Rangpur oil). Well-developed, active and bright male, 28 years, convict overseer, no previous illness except occasional attack of dysentery and constipation. Height 5 ft. 6 in.; weight 128 lb. Pulse 70.

A few small soft glands palpable in the inguinal region.

Total amount of food consumed :—

Dates: 19-9-38 to 18-10-38, missed three days; rice: 466 ounces; oil: 53½ ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms :—
28-9-38. Pain in the waist.
5-10-38. Constipation, after consumption of 28 ounces of oil.
12-10-38. Loose stool and pain in the abdomen.
13-10-38. Loose stool.
24-10-38. Loss of appetite and burning sensation of the finger tips.
29-10-38. Pain in the lower extremities, loss of appetite and constipation.
7-11-38. Burning sensation of the eyes.

Physical signs :—
General appearance : looks slightly reduced.
Weight : 19-9-38, 128 lb.; 7-10-38, 131 lb.; 7-11-38, 128 lb.
Urine : nothing abnormal.

No. 8 (III—'non-saponifiable' fraction of Rangpur oil). Well-developed, active and bright male, 30 years, tape weaver, been in jail for three months. No previous serious illness, complains of inflammation of the right testicle three months back, occasional pain in the right epigastric region and constipation. Height 5 ft. 7 in.; weight 130 lb. Pulse 88.

Total amount of special food consumed :—

Dates: 19-9-39 to 18-10-38, missed three days; rice: 466 ounces; oil: 51 ounces.

Signs and symptoms after commencement of feeding :—

Subjective symptoms :—
20-9-38. Pain in the left testicle.
24-9-38. Uneasiness in the abdomen after the consumption of 12 ounces of oil.
25-9-38. Pain all over the body, especially thigh and calf muscles.
16-10-38. Weakness and loss of appetite.
24-10-38. Loss of appetite.
29-10-38. Burning sensation of the eyes.

Physical signs :—
General appearance : looks slightly reduced.
Weight : 19-9-38, 130 lb.; 7-10-38, 128 lb.; 7-11-38, 126 lb.
20-9-38. Left testicle slightly inflamed and enlarged.
Urine : nothing abnormal.

B. CONTROL GROUP.

No. 10 (III—' jail-produced ' oil). Well-developed, active and bright male, 25 years, convict overseer, been in jail for 13 months. No previous illness. No serious complaint since in jail excepting occasional inflammation of the right testicle and pain in the joints. Height 5 ft. 5 in.; weight 148 lb. Pulse 85.

Total amount of special food consumed :—

   Dates : 19-9-38 to 18-10-38, missed four days; rice : 484 ounces; oil : 55 ounces.

Signs and symptoms after the commencement of the feeding :—

   Subjective symptoms :—
   21-9-38. Headache and loose stools after consuming 4 ounces of oil.

   Physical signs :—
   General appearance : unaltered.
   Weight : 19-9-38, 148 lb.; 7-10-38, 151 lb.; 18-10-38, 152 lb.
   Urine : nothing abnormal.   •

No. 11 (IV—' jail-produced ' oil). Moderately developed, active and bright male, 45 years, works n the stores, been in jail for one year, no previous illness, no complaints since in jail except dimness of vision at night. Height 5 ft. 6¼ in.; weight 147 lb. Pulse 68.

Total amount of food consumed :—

   Dates : 19-9-38 to 18-10-38, missed three days; rice : 304 ounces; oil : 57 ounces.

Signs and symptoms after commencement of feeding, nil.

   Physical signs :—
   General appearance : unaltered.
   Weight : 19-9-38, 147 lb.; 7-10-38, 146½ lb.; 18-10-38, 145 lb.
   Urine : nothing abnormal.

EFFECT OF VISCOSITY OF SERUM ON THE RATE OF ABSORPTION OF ANTIBODIES.

BY

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EVIDENCE has been brought forth recently (Lahiri, 1939) to show that concentrated anti-diphtheritic horse serum is absorbed less rapidly than natural anti-diphtheritic horse serum, when injected intramuscularly or subcutaneously in rabbits. This inferior absorbability of concentrated sera has been ascribed to changes in physico-chemical properties of sera occurring during the processes of concentration and amongst them increased viscosity of concentrated sera has been assumed to be the most important factor.

The deduction on correlation of rise in viscosity with diminution in the rate of absorption of sera from experiments with concentrated sera appears, however, to have some fallacies. As for instance, McFarlane's (*see* Marrack, 1938) investigations with the ultra-centrifuge have shown that horse serum globulin precipitated by half saturation with ammonium sulphate and re-dissolved, exists as particles the greater part of which are uniform and have a weight of about 150,000, but in undiluted serum more than half of serum globulin exists as particles having a weight varying from 70,000 to 150,000. Hence it is quite likely that antibody globulins in a horse serum concentrated by precipitation with ammonium sulphate are, on the average, of a larger size than those present in natural undiluted horse serum.

This possible difference in size between antibody globulins in a concentrated serum and those in a natural serum appears to be of some significance, as evidence has been brought forth (Horsfall *et al.*, 1937) to indicate that rabbit anti-pneumococcus serum, in which the antibody molecules are of a smaller size and weight (Heidelberger and Pedersen, 1937 ; Kabat and Pedersen, 1938) than those in horse serum, may be superior to horse anti-pneumococcus serum as a specific therapeutic agent. Recently, Finland and Brown (1938) have shown that after intramuscular injection of unconcentrated rabbit type I anti-pneumococcus serum in man, the antibody molecules are absorbed more rapidly than when equivalent amounts of concentrated horse serum are injected in the same manner. Presumably the conclusion in their mind was that particles of smaller size were absorbed more rapidly than

those of larger size. However, it may be mentioned in passing that they used un-concentrated rabbit serum against concentrated horse serum and it may be suggested that this difference in the nature of sera, rather than the types of sera, might have been responsible for the observed differences in the rates of their absorption.

This possibility of increase in size of antibody globulin molecules due to frac-tionation with ammonium sulphate and consequently a possible low absorbability of concentrated sera raises some doubt about correctness of the deduction that increase in viscosity of concentrated sera is responsible for a lower rate of absorption of antibodies contained in them, when that deduction is based on the results of experi-ments on absorption of natural serum and that concentrated by fractionation with ammonium sulphate. Moreover, other factors in a concentrated serum, such as the presence of traces of ammonium sulphate or the altered physico-chemical state which may cause inflammation of the tissues with the production of vascular con-gestion and local stasis of circulation and consequently a less rapid liberation of anti-bodies in general circulation, may also falsify the deduction on the effect of viscosity based on experiments with concentrated sera. Menkin (1930) showed that foreign protein injected into an inflamed area was absorbed into the circulation more slowly than when injected into a normal area. Finland and Brown (*loc. cit.*) also laid some stress on this factor as they found that type V concentrated rabbit serum produced moderate local inflammatory reactions in the recipients and absorption in them was delayed as compared with the recipients of concentrated horse serum.

It was, therefore, proposed to compare the rates of absorption of anti-diphtheritic antibodies in horse sera with their viscosities modified in a variety of ways, in order to find out with so much precision as is attainable with the technique followed in these experiments the effect of differences only in viscosity of media containing antibodies on the rates of their absorption.

FIRST EXPERIMENT.

Anti-diphtheritic serum from a horse was freed from suspended red blood corpuscles by centrifuging, 0·5 per cent phenol was added as a preservative and lastly the serum was filtered through Seitz E. K. pads. Its antibody titre as determined using the technique described in a previous paper (Lahiri, *loc. cit.*) was 800 A. U. per c.c.

Gelatin solution was prepared by dissolving 20 g. gelatin flakes in 200 c.c. normal saline, adjusting pH to 7·8, steaming for precipitation of phosphates and finally filtering through fine filter-paper in a steamer. It was sterilized by autoclaving.

For the preparation of serum-gelatin mixture and serum dilution, flasks con-taining serum, gelatin, and sterile normal saline were kept in a water-bath at 40°C. for 5 minutes in order to decrease the viscosity of gelatin sufficiently to allow of a ready mixing with serum and at the same time not destroying the antibodies in serum by excessive heating. In a sterile flask, 40 c.c. of serum and 40 c.c. of gelatin solution were mixed together by gentle agitation. In another flask, 40 c.c. of serum were diluted with 40 c.c. of saline, in order to keep the concentration of antibodies constant in both the substrates. Both the flasks were kept in a water-bath at 40°C. till injections of rabbits were over, so that temperature of fluids injected was

close to that of the body temperature of rabbits. Viscosity of the mixture and the dilution relative to water at 40°C. was determined with the Ostwald viscosimeter.

Rabbits were injected intramuscularly in the thighs with 6,000 A. U. per kg. body-weight. Samples of blood were collected from the heart three hours, 24 hours, and 48 hours after injection. Serum from them was separated and titrated according to the technique described before (Lahiri, *loc. cit.*).

Results are shown in Table I :—

TABLE I.

Comparative rates of absorption of anti-diphtheritic serum mixed with gelatin and that diluted with saline.

	Rabbit number.	Weight of rabbit in g.	Volume injected in c.c.	RATES OF ABSORPTION IN UNITS PER C.C. OF RABBITS' SERUM.		
				3 hours.	24 hours.	48 hours.
Rabbits injected with serum-gelatin mixture.	1	1,700	25·5	9	45	60
Viscosity = 4·07 .. {	2	1,480	22·2	6	45	65
	3	950	14·25	6	40	60
Rabbits injected with serum diluted with saline.	4	1,600	24	6	35	35
Viscosity = 1·65 .. {	5	1,300	19·5	12	60	55
	6	1,280	19·2	15	60	65

Thus antibodies in a less viscous medium such as serum diluted with saline are absorbed during the first twenty-four hours more rapidly than those in medium of higher viscosity such as serum-gelatin mixture. After that period the difference is gradually made up till near about forty-eight hours after injection the level of circulating antibody titre becomes almost equal in all rabbits.

The rate of absorption in rabbit number four was unusually slow. But unfortunately it was old and emaciated and so it is perhaps reasonable to assume that the anatomical nature of its tissues were responsible for the anomaly.

SECOND EXPERIMENT.

Serum used in the first experiment was also used for this experiment. It was stored at 0°C. to 2°C.

Powdered gum acacia sterilized by dry heat was dissolved in 200 c.c. of sterile normal saline in the cold till the relative viscosity of solution became nearly eight. It was then filtered through two layers of sterile muslin.

In a sterile flask 40 c.c. of serum were mixed by gentle shakings with 40 c.c. of gum acacia solution in the cold. In another flask 40 c.c. of serum were diluted with 40 c.c. of sterile normal saline. Both of them were kept in a water-bath at 40°C. for 5 minutes before starting injections and kept there till the injections were over. Viscosities of the mixture and the dilution relative to water at 40°C. were determined.

Rabbits were injected intramuscularly with 6,000 A. U. per kg. body-weight, samples of blood were collected at three hours, 24 hours, and 96 hours after injection, and serum separated and titrated as before.

Results are shown in Table II :—

TABLE II.

Comparative rates of absorption of anti-diphtheritic serum mixed with gum acacia and that diluted with saline.

	Rabbit number.	Weight of rabbit in g.	Volume injected in c.c.	RATES OF ABSORPTION IN UNITS PER C.C. OF RABBITS' SERUM.		
				3 hours.	24 hours.	96 hours.
Rabbits injected with serum-acacia mixture.	1	1,640	24·6	<6	40	30
Viscosity = 4·14 ..	2	1,120	16·8	<6	40	35
	3	890	13·4	6	45	35
Rabbits injected with serum diluted with saline.	4	1,370	20·5	9	60	30
Viscosity = 1·65 ..	5	1,160	17·4	9	60	25
	6	1,100	16·5	12	65	35

The results indicate as in the first experiment that antibodies in a less viscous medium are absorbed during the first twenty-four hours more rapidly than those in a highly viscous medium. Level of circulating antibody titre ninety-six hours after injection is almost the same in all rabbits.

In the first experiment titre after forty-eight hours was found to be almost equal in all rabbits. So it appears that after twenty-four hours the difference in the rates of absorption of antibodies, contained in viscous and less viscous media, is gradually made up till from about forty-eight hours after injection to ninety-six hours after injection or possibly till the complete elimination of antibodies, titre

of circulating antibodies in both groups of rabbits is maintained at the same level.

THIRD EXPERIMENT.

Serum used in this experiment was prepared as in the first experiment. Its antibody titre was 900 A. U. per c.c.

Cream obtained just after skimming cow's milk by centrifugation with aseptic precautions was re-emulsified in 100 c.c. sterile normal saline till the relative viscosity of emulsion was near about eight. It was then filtered through two layers of sterile muslin.

Thirty c.c. of serum were mixed with 30 c.c. of milk-fat emulsion in a sterile flask. In another flask 30 c.c. of serum were diluted with 30 c.c. of sterile normal saline. Their viscosities relative to water at 40°C. were determined. The flasks were maintained at 40°C. during injections.

Rabbits were injected intramuscularly with 6,000 A. U. as usual, blood samples collected at three hours, 24 hours and 48 hours and serum titrated.

Results are shown in Table III :—

TABLE III.

Comparative rates of absorption of anti-diphtheritic serum mixed with milk-fat and that diluted with saline.

	Rabbit number.	Weight of rabbit in g.	Volume injected in c.c.	RATES OF ABSORPTION IN UNITS PER C.C. OF RABBITS' SERUM.		
				3 hours.	24 hours.	48 hours.
Rabbits injected with serum-milk-fat mixture.	1	1,340	17·9	<6	40	60
	2	1,100	14·7	6	40	60
Viscosity = 4·03 ..	3	960	12·8	6	45	55
Rabbits injected with serum diluted with saline.	4	1,260	16·8	9	55	65
	5	1,140	15·2	12	60	60
Viscosity = 1·62 ..	6	940	12·5	12	60	55

Thus, the results of this experiment corroborate the results of the previous experiments.

FOURTH EXPERIMENT.

Serum used in the third experiment was also used for this experiment. It was stored at 0°C. to 2°C.

In a sterile flask, 30 c.c. of concentrated anti-tetanic horse serum with a relative viscosity of 8·5 nearly were mixed with 30 c.c. of anti-diphtheritic serum. In another flask 30 c.c. of anti-diphtheritic serum were diluted with 30 c.c. of sterile normal saline. Both of them were brought up to and maintained at 40°C. in a water-bath during injections. Their viscosities relative to water at 40°C. were determined.

Rabbits were injected with 6,000 A. U. per kg., blood samples collected at three hours, 24 hours, and 48 hours, and serum titrated.

Results are shown in Table IV :—

TABLE IV.

Comparative rates of absorption of anti-diphtheritic serum mixed with concentrated anti-tetanic horse serum and that diluted with saline.

	Rabbit number.	Weight of rabbit in g.	Volume injected in c.c.	RATES OF ABSORPTION IN UNITS PER C.C. OF RABBITS' SERUM.		
				3 hours.	24 hours.	48 hours.
Rabbits injected with serum-concentrated-serum mixture.	1	1,130	15	6	40	55
	2	820	11	6	45	45
Viscosity = 4·75	3	1,130	15	6	40	60
Rabbits injected with serum diluted with saline.	4	1,245	16·6	12	55	60
	5	825	11	12	55	45
Viscosity = 1·62	6	955	12·75	12	60	55

The results are very similar to those of the previous experiments.

Titre in rabbits two and five at forty-eight hours were low comparative to those in rabbits used in other experiments. Low dosage corresponding to low body-weight seems to be a possible cause.

DISCUSSION.

In the four experiments viscosities of natural sera were increased by adding different substances to them, but degrees of increase in viscosity were nearly the same in samples of all the four experiments. Viscosities of diluted sera used as

controls were also almost the same in all the experiments. Hence results of the four experiments may be considered together for deducing a conclusion. They indicate that antibodies in a medium of high viscosity are absorbed less rapidly than those in a medium of lesser viscosity.

Difference in the rates of absorption of antibodies in a viscous medium and those in a less viscous medium appear to be gradually made up till from about forty-eight hours after injection the titre of antibodies absorbed in the circulation reach the same level in both cases, and they remain equal ninety-six hours after injection and possibly afterwards too. This happens perhaps due to dilution of a highly viscous medium injected in a tissue by fluids gradually drawn into the area by high colloid osmotic pressure of the medium till its osmotic pressure is in equilibrium with that in the vessels in the neighbourhood. Possibly thus the viscosity is diminished sufficiently to allow an optimum absorption of antibodies after a time.

Thus, it appears that high viscosity of a therapeutic serum is detrimental to so much of its therapeutic efficacy as is due to rapid absorption of antibodies during the first twenty-four hours after injection. After that period the rates of absorption of antibodies in both viscous and less viscous sera appear to equal, and if antibodies in a particular medium or in a particular physico-chemical state are not poorer therapeutically, their therapeutic efficacies are also likely to be of the same degree after that period.

Thus, these experiments seem to demonstrate with some clarity the effect of viscosity of media containing antibodies on the rate of their absorption. There was possibly no cause for gross differences in particle size of antibody molecules in experimental and control media, though this cannot be asserted without actual determinations of particle size of antibody molecules in different media experimented with. However, these experiments do not in any way exclude the possibility that particle size may determine the rate or degree of absorption of antibodies.

It does not appear to be likely that different substances in several experimental viscous media produced the same degree of inflammation of the tissues injected, so as to retard liberation of antibodies in the general circulation to the same exent in all the four experiments. Moreover, it is difficult to imagine that inflammatory changes brought about by those substances after their introduction in tissue in a large bulk, should disappear twenty-four hours after their injection, so as to allow the rates of absorption of antibodies in experimental and control media to equal.

SUMMARY.

Antibodies in anti-diphtheritic horse serum are absorbed less rapidly after intramuscular injection in rabbits when they are mixed with substances such as gelatin, gum acacia, milk-fat, or concentrated anti-tetanic horse serum in order to increase their viscosities, than when they are diluted with saline. Difference in the rates of absorption is gradually made up till from near about forty-eight hours after injection the rates become equal.

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STUDIES ON FILARIASIS TRANSMISSION.*

BY

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Fund Association at the School of Tropical Medicine, Calcutta.)*

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THE object of the present investigation is the determination of the various factors responsible for the transmission of filariasis by *Culex fatigans*. The present communication gives an account of the observations made on the influence of some factors on the degree of infection of *Culex fatigans* with the embryos of *W. bancrofti* as the result of experimental feeding on the blood of filariasis patients. The influence of the following factors were studied: (*a*) effect of varying atmospheric temperature and relative humidity, (*b*) effect of density of infection in the blood of the donors, and (*c*) influence of age and race of donors.

SUMMARY OF PREVIOUS WORK.

Sundar Rao and Iyengar (1930) studied the infection of *Culex fatigans* with *Wuchereria bancrofti* in different seasons in Calcutta. In the monsoon they found that the heaviest infection and complete development of the embryos took place in 10 to 11 days. During winter the infection rate was very slow and complete development of the embryos took place in 18 to 20 days. During the hot season the infection rate was low and it took 11 to 12 days for complete development of the embryos. They further observed the behaviour of two batches of mosquitoes fed on the same case during the month of February, one of which was kept at room temperature, while the other batch was kept in a wet incubator in which a high humidity and a moderate temperature was maintained. In the former there was extremely slow development, the infection rate was very low, and the number of embryos very small. In the latter the infection was heavy and the development was more rapid.

Acton and Sundar Rao (1931) observed that, in India, filarial infections occur along the coast and course of the great rivers except the Indus. They laid down certain factors which determine whether the area is hyper-endemic, endemic, or of low endemicity.

Sundar Rao and Iyengar (1932) observed the experimental infection of some Indian mosquitoes with *W. bancrofti*. *Culex fatigans, Anopheles philippinensis, A. pallidus, A. annularis, A. stephensi,* and *A. ludlowii* var. *sundaica* were found to be efficient intermediate hosts of *W. bancrofti*.

Knowles and Basu (1934) studied the mosquito prevalence and mosquito-borne diseases in Calcutta city. They observed that the intensity of breeding of *Culex fatigans* is at a very low level during the monsoon, the most favourable period of transmission. The peak of *Culex* breeding is in

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November when conditions for filariasis transmission rapidly become unfavourable. This want of coincidence keeps the filariasis rate at a relatively low level.

Hu (1935) found infective larvæ of *W. bancrofti* to be able to survive over 79 days in their mosquito host, in addition to an incubation period of 14 days in *Culex pipiens* var. *pallens* of the Shanghai area. The average mean room temperature and humidity during this series of observation were 76·2°F. and 77·9 per cent, respectively, for the 14 days of incubation of the parasites and 66°F. and 74·7 per cent for the rest of 93 days after the time of infection of the mosquitoes.

Knowles, Basu and Sundar Rao (1936) published a summary report on filariasis transmission in relation to atmospheric temperature and relative humidity.

Hu (1937) studied the influence of microfilarial density of the blood on the infection in the mosquitoes. He found the rate of infection was higher when they were fed on heavy filarial cases than when they fed on light filarial cases.

PRESENT OBSERVATIONS.

Batches of clean laboratory-bred *Culex fatigans* were fed on suitable cases of filariasis (with filarial embryos in the peripheral blood) between 10 p.m. and 11 p.m. put up in Barraud Cages and exposed to 36 different combinations of temperature and relative humidity in the air-conditioning cabinet at which any temperature between 50°F. and 100°F. and relative humidity between 50 and 100 per cent can be adjusted. In each case a microfilaria count of the patient's blood was made before the feed in order to ascertain the influence of density of microfilaria in donor's blood on infectivity to mosquitoes. The survivors were dissected after varying intervals of time to test their infectivity.

TABLE I.

Temperature and filariasis transmission.

Temperature in °F.	Relative humidity in per cent.	Experiment.	Number of mosquitoes fed.	Number of mosquitoes survived.	Filaria count per 0·2 c.c.	Thorax infection.	Proboscis infection.	Total infection.	Interval between feed and examination in days.
50	50	1	250	132	..	0	0	0	12—22
50	60	2	250	83	..	0	0	0	9—16
50	70	1	250	90	..	0	0	0	9—16
50	80	1	250	155	..	0	0	0	11—21
50	90	1	250	70	..	0	0	0	9—13
50	100	1	253	62	..	0	0	0	9—15
50	TOTALS .	7	1,503	592	358—410	0 (0 per cent.)	0 (0 per cent.)	0 (0 per cent.)	9—22

TABLE I.—*contd.*

Temperature in °F.	Relative humidity in per cent.	Experiment.	Number of mosquitoes fed.	Number of mosquitoes survived.	Filaria count per 0·2 c.c.	Thorax infection.	Proboscis infection.	Total infection.	Interval between feed and examination in days.
60	50	3	240	207	..	0	0	0	14—21
60	60	4	269	193	..	0	0	0	12—24
60	70	2	145	117	..	11	0	11	16—21
60	80	2	388	202	..	3	4	4	12—47
60	90	2	280	169	..	3	0	3	12—23
60	100	2	335	148	..	8	0	8	6—18
60	TOTALS .	15	1,657	1,036	7—217	25 (2·4 per cent.)	4 (0·4 per cent.)	26 (2·5 per cent.)	6—47
70	50	3	358	64	..	0	0	0	14—17
70	60	2	250	85	..	0	0	0	13—15
70	70	3	238	191	..	86	27	86	7—27
70	80	3	153	112	..	77	36 (hæmocœle 3)	78	10—30
70	90	3	290	216	..	28	0	28	11—22
70	100	4	362	220	..	89	26 (hæmocœle 4)	89	5—33
70	TOTALS .	18	1,651	888	7—560	280 (31·5 per cent.)	89 (hæmocœle 7) (10 per cent.)	281 (31·6 per cent.)	5—33

TABLE I—*concld.*

Temperature in °F.	Relative humidity in per cent.	Experiment.	Number of mosquitoes fed.	Number of mosquitoes survived.	Filaria count per 0·2 c.c.	Thorax infection.	Proboscis infection.	Total infection.	Interval between feed and examination in days.
80	50	3	440	50	..	0	0	0	5—7
80	60	2	283	69	..	7	0	7	7
80	70	2	278	78	..	37	0	37	7
80	80	2	217	83	..	45	0	45	7
80	90	6	438	63	..	42	46	63	6—31
80	100	4	325	132	..	96	34	96	6—31
80	TOTALS .	19	1,981	475	26—140	227 (47·7 per cent.)	80 (16·8 per cent.)	248 (52·2 per cent.)	5—31
90	50	3	322	0	..	..	..	..	3—4
90	60	3	186	14	..	0	0	0	8—11
90	70	2	130	50	..	0	0	0	5—7
90	80	1	144	77	..	28	19	33	5—12
90	90	4	447	186	..	85	41	85	6—11
90	100	3	233	77	..	52	34	52	8—17
90	TOTALS .	16	1,462	404	20—150	165 (40·8 per cent.)	94 (23·2 per cent.)	170 (42 per cent.)	3—17
100	50	1	217	0	..	..	..	..	3
100	60	1	250	0	..	..	..	..	2
100	70	1	150	0	..	..	..	..	3
100	80	2	192	0	..	..	..	..	2—4
100	90	3	257	0	..	..	..	..	1—3
100	100	1	252	0	..	..	..	..	3
100	TOTALS .	9	1,318	0	56—198	..	..	..	1—4

Table I and Graph 1 represent the percentages of infectivity of *C. fatigans* with *W. bancrofti* and their relation to atmospheric temperature. It is seen that at a temperature of 50°F. there is no infection. Proboscis infection rises steadily from

GRAPH 1.

TEMPERATURE AND FILARIASIS TRANSMISSION.

Showing the rate of infection in the experimentally-fed mosquitoes in relation to temperature.

Temperature °F.

Percentages in infectivity.
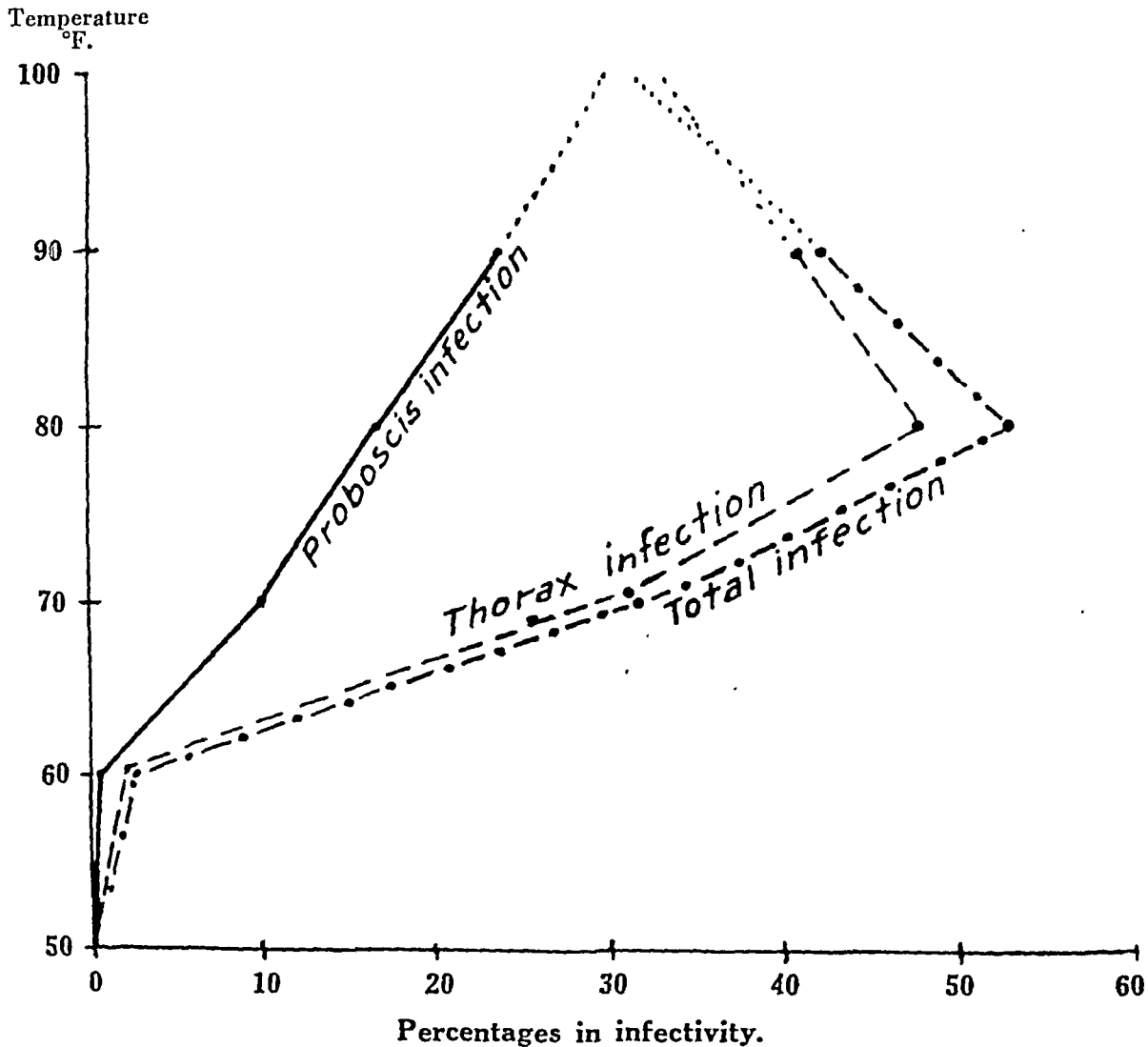
60°F. to 90°F. but thorax and total infections rise steadily up to 80°F. and then there is a slight fall at 90°F. At 100°F. mosquitoes will not survive till infection.

TABLE II.

Relative humidity and filariasis transmission.

Relative humidity in per cent.	Temperature in °F.	Experiment.	Number of mosquitoes fed.	Number of mosquitoes survived.	Filaria count per 0·2 c.c.	Thorax infection.	Proboscis infection.	Total infection.	Interval between feed and examination in days.
50	50	1	250	132	..	0	0	0	12—22
50	60	3	240	207	..	0	0	0	14—21
50	70	3	358	64	..	0	0	0	14—17
50	80	3	440	50	..	0	0	0	5—7
50	90	3	332	0	..	..	..	..	3—4
50	100	1	217	0	..	..	..	..	1—4
50	Totals .	14	1,837	453	7—358	0 (0 per cent.)	0 (0 per cent.)	0 (0 per cent.)	1—22
60	50	2	250	83	..	0	0	0	9—16
60	60	4	269	193	..	0	0	0	12—24
60	70	2	250	85	..	0	0	0	13—15
60	80	2	283	69	..	7	0	7	7
60	90	3	186	14	..	0	0	0	8—11
60	100	1	250	0	..	..	..	..	2
60	Totals .	14	1,488	444	53—410	7 (1·5 per cent.)	0 (0 per cent.)	7 (1·5 per cent.)	2—24

TABLE II—*contd.*

Relative humidity in per cent.	Temperature in °F.	Experiment.	Number of mosquitoes fed.	Number of mosquitoes survived.	Filaria count per 0·2 c.c.	Thorax infection.	Proboscis infection.	Total infection.	Interval between feed and examination in days.
70	50	1	250	90	..	0	0	0	9—16
70	60	2	145	117	..	11	0	11	16—21
70	70	3	238	191	..	86	27	86	7—27
70	80	2	278	78	..	37	0	37	7
70	90	2	130	50	..	0	0	0	5—7
70	100	1	150	0	..	..	..	..	3
70	TOTALS .	11	1,191	526	12—410	134 (25·4 per cent.)	27 (5·1 per cent.)	134 (25·4 per cent.)	3—27
80	50	1	250	155	..	0	0	0	11—21
80	60	2	388	202	..	3	4	4	12—47
80	70	3	153	112	..	77	36	78 (hæmocœle 3)	10—30
80	80	2	217	83	..	45	0	45	7
80	90	1	144	77	..	28	19	33	5—12
80	100	2	192	0	..	..	..	..	..
80	TOTALS .	11	1,344	629	59—358	153 (24·3 per cent.)	59 (hæmocœle 3) (9·3 per cent.)	160 (25·4 per cent.)	5—47

TABLE II—*concld.*

| Relative humidity in per cent.	Temperature in °F.	Experiment.	Number of mosquitoes fed.	Number of mosquitoes survived.	Filaria count per 0·2 c.c.	Thorax infection.	Proboscis infection.	Total infection.	Interval between feed and examination in days.
90	50	1	250	70	..	0	0	0	9—13
90	60	2	280	169	..	3	0	3	12—23
90	70	3	290	216	..	28	0	28	11—22
90	80	6	438	63	..	42	46	63	6—31
90	90	4	447	186	..	85	41	85	6—11
90	100	3	257	0	..	..	..	..	1—3
90	TOTALS .	19	1,962	704	28—410	158 (22·4 per cent.)	87 (12·2 per cent.)	179 (25·4 per cent.)	1—31
100	50	1	253	62	..	0	0	0	9—15
100	60	2	335	148	..	8	0	8	6—18
100	70	4	362	220	..	89	26 (hæmocœle 4)	89	5—33
100	80	4	325	132	..	96	34	96	6—31
100	90	3	233	77	..	52	34	52	8—17
100	100	1	252	0	..	..	..	..	3
100	TOTALS .	15	1,760	639	26—410	245 (38·3 per cent.)	94 (hæmocœle 4) (14·7 per cent.)	245 (38·3 per cent.)	3—33

Table II and Graph 2 show the relation between relative humidity and infectivity of *C. fatigans* with *W. bancrofti*. At a relative humidity of 50 per cent and any temperature between 50°F. and 100°F. no infection of the mosquitoes was observed.

GRAPH 2.

RELATIVE HUMIDITY AND FILARIASIS TRANSMISSION.

Showing the rate of infection in the experimentally-fed mosquitoes in relation to relative humidity.

Relative humidity in per cent.
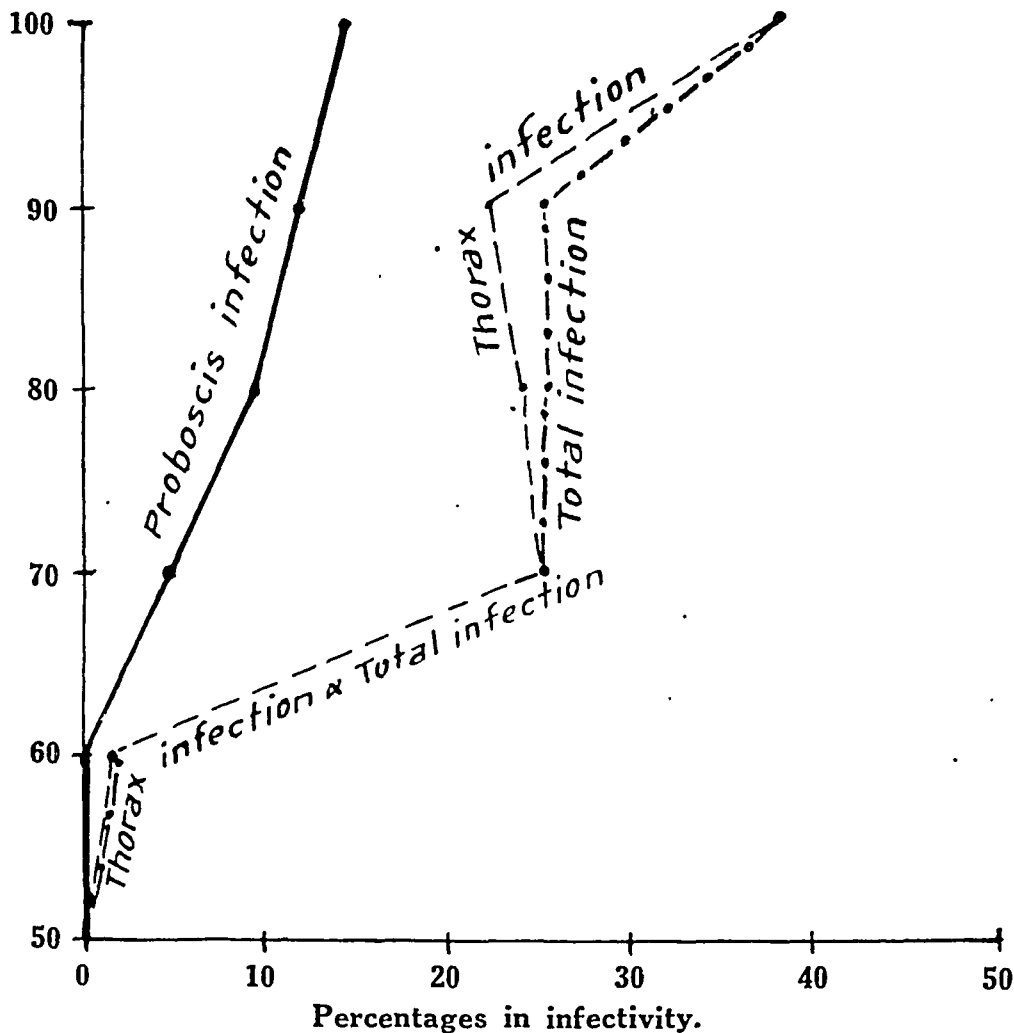
At 60 per cent relative humidity with favourable temperature a very poor percentage of infectivity was observed. At 70 per cent relative humidity with favourable temperature a good percentage of thorax and total infection was observed though the proboscis infection rate was very low at this relative humidity.

Proboscis, thorax and total infection reached their peaks at a relative humidity of 100 per cent. From 70 per cent humidity infection rate was progressive up to 100 per cent humidity if the slight fall of thorax and total infection at 90 per cent humidity is accounted for as an experimental error.

<center>TABLE III.</center>

Atmospheric temperature and relative humidity—Filariasis transmission.

Temperature in °F.	Relative humidity in per cent.					
	50	60	70	80	90	100
50	0	0	0	0	0	0
60	0	0	+	+	+	+
70	0	0	++	+++	+	++
80	0	+	++	+++	++++	+++
90	0	0	0	++	++	+++
100	0	0	0	0	0	0

<center>TABLE IV.</center>

Atmospheric temperature and relative humidity—Filariasis transmission.

Temperature in °F.	Relative humidity in per cent.					
	50	60	70	80	90	100
50	0	0	0	0	0	0
60	0	0	9·4	1·9	1·8	5·4
70	0	0	45	69·6	12·9	40·4
80	0	10·1	47·4	54·2	100	72·7
90	×	0	0	42·8	45·6	67·5
100	×	×	×	×	×	×

Tables III and IV represent the infectivity of mosquitoes at different combinations of temperature and humidity. In Table III

+	indicates infectivity between	1 and	25 per cent.
++	,,	,,	,,	26 ,,	50	,,
+++	,,	.,	,,	51 ,,	75	,.
++++	,,	,.	,,	76 ,,	100	,,

In Table IV the numbers are percentages of infectivity among the surviving mosquitoes. It is clear that the area within the thick line is the danger zone, i.e., temperature between 70°F. and 90°F. and humidity between 70 and 100 per cent.

TABLE V.

TIME AND DEVELOPMENT OF MICROFILARIA.

Showing the minimum time (in days) taken for the complete development of microfilaria in mosquitoes and their appearance in the proboscis.

Temperature in °F.	Relative humidity in per cent.					
	50	60	70	80	90	100
50	—	—	—	—	—	—
60	—	—	? Immature forms in the thorax in 19 days.	47	? Immature forms in the thorax in 17 days.	? Immature forms in the thorax in 16 days.
70	—	—	23	20	21	20
80	—	? Mature embryos in the thorax in 7 days.	? Mature embryos in the thorax in 7 days.	? Mature embryos in the thorax in 7 days.	10	10
90	—	—	—	9	9	10
100	—	—	—	—	—	—

— indicates there was no infection at all. ? indicates there was infection but the time for complete develo[p]ent was not obtained.

Table V represents the minimum time taken for the complete development of the microfilaria in *C. fatigans* and their appearance in the proboscis of the mosquitoes. It is observed that temperature has a great influence over it. At a temperature of 90°F. it will take 9 days ; at 80°F. it will take 10 days ; at 70°F., 20 days ; whereas at 60°F. it will take 47 days for the complete development of the embryos.

TABLE VI.

Microfilaria count and filariasis transmission.

Filaria count per 0·2 c.c.	Number of mosquitoes fed.	Number of mosquitoes survived.	Experiment.	Thorax infection.	Proboscis infection.	Total infection.	Interval between feed and examination in days.
1— 50 ..	1,226	438	15	82 (18·7 per cent.)	54 (12·3 per cent.)	84 (19·1 per cent.)	3—28
51—100 ..	3,440	995	31	232 (23·3 per cent.)	78 (7·8 per cent.)	255 (25·6 per cent.)	1—47
101—150 ..	1,416	611	12	256 (41·8 per cent.)	105 (17·1 per cent.)	259 (42·3 per cent.)	3—31
151—200 ..	1,229	252	10	43 (17 per cent.)	12 (4·7 per cent.)	43 (17 per cent.)	2—24
201—300 ..	606	400	6	74 (18·5 per cent.)	18 (4·5 per cent.)	74 (18·5 per cent.)	5—23
301—600 ..	1,655	699	9	10 (1·4 per cent.)	0 (0 per cent.)	10 (1·4 per cent.)	9—22

Graph 3 and Table VI represent the infectivity of *C. fatigans* with *W. bancrofti* in relation to the density of microfilaria in the peripheral blood of the donor on

whom mosquitoes were fed. The infectivity curve reaches its peak when the mosquitoes were fed on the donor with 101 to 150 microfilaria per 0·2 c.c. blood. But when the count was increased or decreased the infectivity curve fell on

GRAPH 3.

MICROFILARIA COUNT AND FILARIASIS TRANSMISSION.

Showing the rate of infection of mosquitoes in relation to microfilaria count.
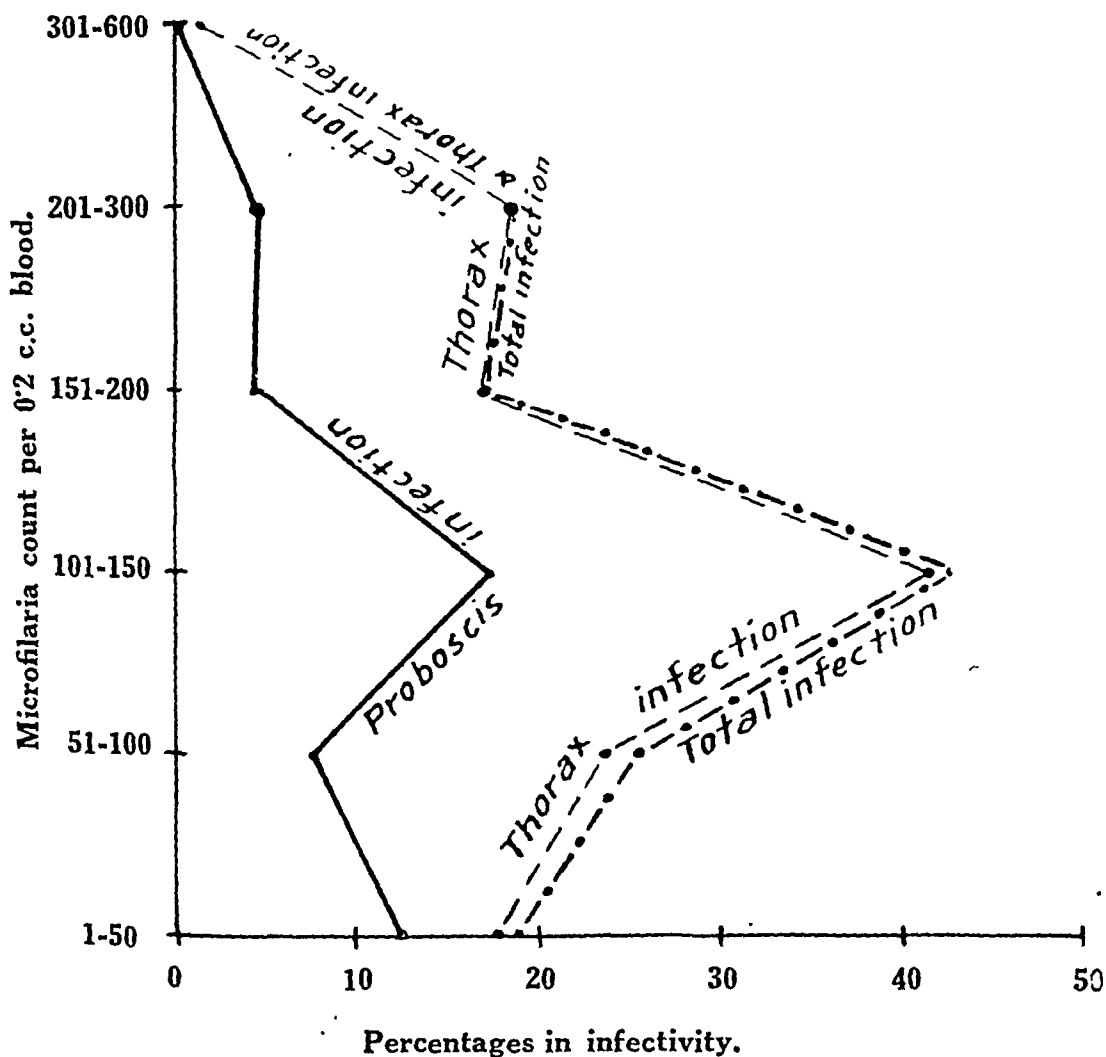
both the ways. It is likely that when the density of the microfilaria is above 150 per 0·2 c.c. they die in mosquito's gut due to overcrowding. It is very

interesting that with the highest microfilaria count we found the lowest infectivity rate. We feel that Graph 3 is self-explanatory.

TABLE VII.

Age of the donor and filariasis transmission.

Age of the donor in years.	Experiment number.	Filaria count.	Number of mosquitoes survived.	Thorax infection.	Proboscis infection.	Total infection.
11—20 ..	20	7—140	655	207 (31·6 per cent.)	60 (9·1 per cent.)	207 (31·6 per cent.)
21—30 ..	54	12—560	2,692	650 (24·1 per cent.)	267 (9·9 per cent.)	659 (24·4 per cent.).
31—40 ..	23	15—213	885	179 (20·2 per cent.)	89 (10 per cent.)	184 (20·9 per cent.)
41—50 ..	7	7— 57	89	25 (28 per cent.)	25 (28 per cent.)	25 (28 per cent.)
51—60 ..	16	40—198	73	39 (53·4 per cent.)	46 (63 per cent.)	60 (82·1 per cent.)

Graph 4 and Table VII represent the infectivity of *C. fatigans* with *W. bancrofti* with relation to the age of the patients on whom mosquitoes were fed. Almost uniform rate of infectivity was observed up to the 40th year of age of the patient. From the 40th year the infectivity rate is directly proportional to the age. the highest peak of infectivity being observed at the age of 51 to 60. There must be some physiological factor which is responsible for this phenomenon.

GRAPH 4.

AGE OF THE DONOR AND FILARIASIS TRANSMISSION.

Showing the relation of the mosquito infection to the age of the donor.
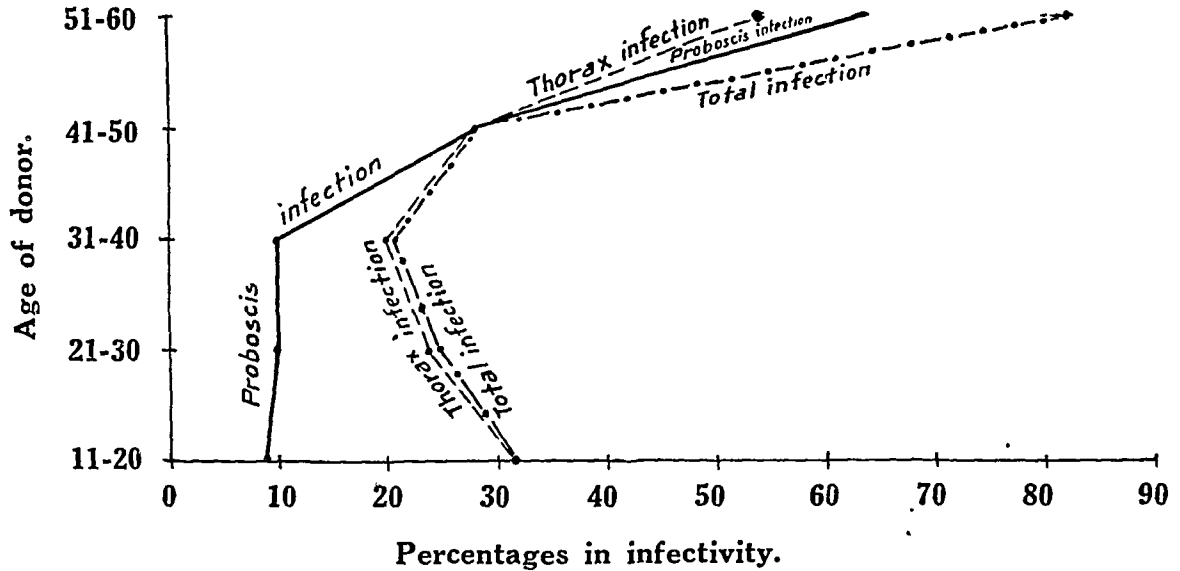
TABLE VIII.

Caste and filariasis transmission.

Caste.	Experiment.	Filaria count.	Number of survivals.	Thorax infection.	Proboscis infection.	Total infection.
Hindu	69	7—560	2,677	521 (19·4 per cent.)	244 (9·1 per cent.)	551 (20·5 per cent.)
Mohammedan ..	41	7—198	1,423	444 (31·2 per cent.)	182 (12·7 per cent.)	451 (31·6 per cent.)
Indian Christian ..	10	43—87	294	132 (44·8 per cent.)	63 (21·4 per cent.)	132 (44·8 per cent.)

Indian Christians seem to be most infective to mosquitoes, then come the Mohammedans and lastly the Hindus.

SUMMARY.

Batches of clear laboratory-bred *Culex fatigans* were fed on suitable cases of filariasis (with embryos of *W. bancrofti* in the peripheral blood) between 10 p.m. and 11 p.m. and immediately after feeding were exposed to 36 different combinations of atmospheric temperature and relative humidity.

Both temperature and relative humidity play a very important part in the transmission of *W. bancrofti* in man by *Culex fatigans.*

At a temperature of 100°F. with relative humidity between 50 and 100 per cent transmission of filariasis does not seem possible as the mosquitoes do not survive till the infective period.

At 90°F. and relative humidity between 50 and 70 per cent no infection was seen in mosquitoes; but at the same temperature and high humidities between 80 and 100 per cent a fairly heavy percentage of infectivity among the survived mosquitoes was observed.

At 80°F. and 90 and 100 per cent relative humidities, a very heavy percentage of mosquitoes become infected, in fact 100 per cent infection was seen at 80°F. and 90 per cent humidity. Thus this seems to be the optimum condition.

At 70°F. and humidities between 70 to 100 per cent a fairly heavy percentage of infection has been seen but at the same temperature and lower humidities no infection has been seen.

At 60°F. and between 70 and 100 per cent relative humidities, a very poor percentage of mosquitoes become infected, and at the same temperature and lower humidities none were infected. At lower temperature the longevity of mosquitoes increases but the minimum time taken for the complete development of the filarial embryos is very long; thus transmission in nature is doubtful in lower temperature.

The minimum time taken for the complete development of the filarial embryos and their appearance in the proboscis of the mosquitoes was 9, 10, 20, and 47 days at 90°F., 80°F., 70°F., and 60°F. temperatures, respectively.

A minimum of 12 microfilaria per 0·2 c.c. blood has been found infective to mosquito. Highest infection was observed when microfilaria count was 101 to 150 per 0·2 c.c. blood. Below and above this count the infectivity decreases.

Age of the donor is very important in mosquito infectivity. From the 40th year the infectivity rate is directly proportional to the age, the highest peak of infectivity being observed at the age of 51 to 60.

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TICKS FOUND ON MAN.

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ALTHOUGH Covell's (1936) and Covell and Mehta's (1936) works on the transmission of certain typhus-like fevers indicate the flea as a natural vector, the possibility of the tick being also a vector as suggested by Megaw (1924) must be borne in mind. It will be remembered that Zinsser (1935) considered the flea to be the natural vector of Brill's disease and that the louse was somewhat of an interloper in the transmission of typhus. We, therefore, submit the schedule of those ticks found in relation to man of which we have records.

The following is our record :—

Locality.	*Identification.*
Calcutta	*Amblyomma (nymph).*
Calcutta	*Rhipicephalus sanguineus.*
Calcutta	*Dermacentor (nymph).*
24 Parganas (Bengal)	*Dermacentor (nymph).*
Naihati (Bengal)	*Amblyomma (nymph).*
Ambootia (Darjeeling Hills)	*Rhipicephalus sanguineus.*
Darjeeling	*Ixodes acutitarsus.*
Bhimtal (Kumaon Hills)	*Rhipicephalus sanguineus.*
Bhimtal (,,)	*Hæmaphysalis montgomeryi.*
Bhawali Ramgarh (Kumaon Hills)	*Rhipicephalus hæmaphysaloides.*
Saugor (Central Province)	*Hyalomma ægyptium.*
Chitora (Central Province)	*Hyalomma ægyptium.*
Chitora ,,	*Hæmaphysalis Sp. (nymph).*
Nowgong (Assam)	*Hæmaphysalis bispinosa.*
Bangalore	*Hæmaphysalis aculeata.*
Lucknow	*Rhipicephalus sanguineus.*
Not known	*Amblyomma Sp. (nymph).*

The specimens were all captured on man and in many instances were reported as having bitten him and our identifications have been checked or corrected by Mr. Cecil Warburton of Cambridge to whom we are greatly indebted.

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HÆMATOLOGICAL STUDIES IN INDIANS.

Part X.

MEAN RED CELL DIAMETERS: STANDARD CURVES FOR A BENGALEE POPULATION.

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PRICE-JONES (1933) measured the mean diameter of the 500 red cells of each of 100 healthy individuals. From these figures he constructed two curves which he called, respectively, the ideal curve for the smallest and the ideal curve for the largest, mean diameter within normal limits. These two curves have been accepted by hæmatologists in Great Britain and America as the standards against which the curves obtained in different anæmic states are judged.

The object of the present investigation is to provide two similar curves based on data obtained from Indians. There is of course every probability that there will be differences between Indian populations, that is, between people living in different parts of India, at different heights above sea levels, of different social statuses, and with different dietary habits. In this paper we are only concerned with people living in Calcutta; the subjects were 50 healthy Bengalee Hindu males between the ages of 18 and 53, doctors, students. laboratory assistants and servants attached to. or associated with, the All-India Institute of Hygiene or the School of Tropical Medicine. The volunteers were selected only from those who had not suffered from dysentery, malaria or any continuous fever. or kala-azar.

The height, weight and age of each was recorded. The finger blood was taken to make the smears, and in each instance it was taken in the forenoon.

The method adopted for measuring the mean diameters is a modification of the method of Hynes and Martin (1936) which was devised by one of the present writers (Sankaran and Rao, 1938). Each cell was measured to the nearest 0.25μ. Details of this method, the great advantage of which is that 500 cells can be measured in about an hour, are given in the latter paper. The data are given in the *Appendix*.

STATISTICAL ANALYSIS OF DATA.

The objects of this statistical analysis are :—

    (1) To estimate standard value for mean red-blood cell diameters for Bengalee persons, and

    (2) To determine the chance variation which may normally be expected in the standard mean diameter.

Information relating to 50 different persons who according to their clinical examination were believed to be normal healthy persons has been recorded ; in the *Appendix* are set out the frequency distributions of about 500 red-blood-cell-diameter measurements on each of these fifty persons. The mean red-blood-cell diameters are also shown in the same table.

It will be seen that there is a considerable variation between different individuals in the mean diameters, the range of variation being from 6.721μ to 7.978μ. Moreover, the variation or scatter exhibited by each of the fifty frequency distributions is smaller than that of the total frequency distribution in the last column. The variation observed in the total frequency distribution may be considered to be partly due to differences in the mean values and partly to the variation noticed in each frequency distribution. The question which arises therefore is whether the variation in the mean diameters can be reasonably neglected, or whether the fifty persons differ in regard to mean diameters, from each other, to such an extent as to form a heterogeneous set of measurements. For the purpose of determining a standard measurement of mean cell diameter, it is essential that the estimation should be based on data which can be considered to be homogeneous. It cannot be said with certainty that a clinical examination alone was sufficient for securing such a homogeneous set of data. Many other unrecognized considerations affecting the cell diameters in the human volunteers may have entered. Further, there may have been introduced in the data errors in actual recording and grouping.

An examination has, therefore, first of all been made of the homogeneity of the available material. As mentioned above, the total variation exhibited in the table may be considered to belong to two separate categories :—

    (*a*) Variation exhibited by measurement for each person.

    (*b*) Variation from person to person.

Variation (*a*) is essentially due to uncontrollable factors and is inherent in the data. This, therefore, is taken as an estimate of variation which may be expected

to occur as a result of chance and is called the 'residual'. If we take the point of view that the normal red-cell diameters of a single species should all be the same, except for the inherent or residual variations, then we may argue that variation (b) should be of the same order as variation (a). If, however, variation (b) is comparatively greater than (a) then we may postulate that there is indication of heterogeneity.

With the help of analysis-of-variance technique, it is possible to measure numerically the variations due to causes (a) and (b). This has been done in Table I which analyses the total variation into two parts corresponding to (a), called the 'residual' and corresponding to (b), called the 'between persons':—

TABLE I.

Analysis of variance of data presented in the Appendix.

Source of variation.	Degrees of freedom.	Sum of squares.	Mean variances.	Ratio of variance.
Between persons ..	49	1,531·2590	31·2502	142·38 (significant).
Residual ..	26,486	5,813·2600	0·2195	..
TOTAL ..	26,535	7,344·518994	0·276786	..

A statistical test of significance applied to the mean variances calculated in this table shows there is 'between persons' a significantly greater variability than can be accounted for by chance. The data must, therefore, be considered to be heterogeneous and an estimate of mean diameter from this table may not be reliable.

An attempt has therefore to be made to eliminate those persons who are the cause of this heterogeneity. Apart from the measurement of cell diameters, information in regard to age, height and weight of the persons was collected. This information is set out in Table II :—

TABLE II.

Data relating to age, weight, and height of volunteers.

Serial number.	Mean diameter.	Age.	Height in inches.	Weight in pounds.
1	7·370	30	67	146
2	6·880	30	64	134
3	7·530	24	68	116

Table II—*contd.*

Serial number.	Mean diameter.	Age.	Height in inches.	Weight in pounds.
4	6·840	28	68	154
5	7·036	35	70	125
6	7·114	35	65	116
7	7·586	30	66	144
8	7·100	23	69	160
9	7·738	24	65	127
10	6·721	24	68	114
11	6·864	31	68	140
12	7·290	27	64	114
13	7·434	38	67	144
14	7·293	28	65	119
15	7·462	25	69	112
16	7·580	25	65	123
17	7·107	33	66	136
18	7·364	27	66	113
19	7·110	28	64	120
20	7·434	21	68	114
21	7·030	24	68	105
22	7·206	23	64	110
23	7·532	28	63	138
24	6·919	29	66	154
25	7·285	36	64	160
26	7·267	25	70	167
27	7·216	26	68	132
28	7·076	28	66	132
29	7·010	19	63	120
30	7·313	24	64	104
31	7·079	22	66	129

TABLE II—*concld.*

Serial number.	Mean diameter.	Age.	Height in inches.	Weight in pounds.
32	6·738	25	65	143
33	7·451	28	54	133
34	7·633	28	66	112
35	7·249	26	64	110
36	7·280	20	64	105
37	7·460	29	69	154
38	7·573	22	67	119
39	7·205	53	66	128
40	7·470	26	65	154
41	7·655	33	64	112
42	7·978	20	64	99
43	6·790	32	71	140
44	7·495	27	63	123
45	7·553	30	70	132
46	7·498	30	65	100
47	7·603	18	62	100
48	7·072	30	66	113
49	7·360	41	67	127
50	7·430	28	67	117

It was thought that if any relationship existed between mean diameter and any of these factors it could be taken as a basis for the elimination of some of the widely divergent cases. For this purpose partial co-efficients of correlation were worked out as follows. If the numbers 1, 2, 3, 4, denote mean diameter, age, height, and weight, respectively, and we denote as usual by symbol $r_{12\cdot34}$ the co-efficient of correlation between variables 1 and 2 on the assumption that variables 3 and 4 do not vary, then it is found that

$$r_{12\cdot34} = -0\cdot044$$

$$r_{13\cdot24} = -0\cdot180$$

$$r_{14\cdot23} = -0\cdot215$$

These three correlations denote, respectively, the degree of independent association between mean diameters, and age, height, and weight. All these values are insignificantly small and, therefore, afford no clue for securing homogeneity of the material. Some other method of eliminating heterogeneous element had therefore to be tried.

There are several methods for eliminating outlying observations but the method described below appears to be adequate in this case. A fairly reliable estimate of the expected chance variation is already available from the residual value from Table I. On the basis of this value it can be estimated that, if the number of cells is 500, the standard error of the mean value should be $\sqrt{\frac{0 \cdot 219484}{500}} = 0 \cdot 020952$. If this standard error is multiplied by 5·152, we get a range of variation within which 99 per cent of the mean values must lie if they all belong to a homogeneous set.

A frequency distribution of the 50 mean values was worked out and is set out in Table III :—

<div align="center">TABLE III.</div>

Frequency distribution of the mean values of fifty persons and the expected distribution from the fitted curve.

Mean diameter.	Observed frequency.	Frequency expected from the fitted curve.	
6·6875—	3	1	
6·8125—	3	2	3·571
6·9375—	6	4	
7·0625—	5	6	0·167
7·1875—	9	8	0·125
7·3125—	8	8	0·000
7·4375—	8	8	0·000
7·5625—	6	6	0·000
7·6785—	1	4	
7·8125—	..	2	3·571
7·9375—	1	1	

This distribution was smoothed with the help of Pearsonian curve type I. The equation of this curve was found to be

$$Y = 8 \cdot 5147 \left[1 + \frac{X}{8 \cdot 0564} \right]^{5 \cdot 4473} \left[1 - \frac{X}{8 \cdot 7842} \right]^{5 \cdot 9394}$$

The expected values from this equation are also shown in Table III. The value of χ^2 to test the goodness of fit of this curve to actual data was found to be 7·43, which suggests that the fit may be considered to be satisfactory. The curve has undoubtedly given a good fit to the middle portion of the distribution, but towards both the tails the fit is unsatisfactory. This is perhaps due to the heterogeneity.

The modal value, i.e., the value which would occur most frequently, was calculated from the equation of this curve and was found to be 7·2918. If round this value we fix deviations ± 2·576 × 0·020952, i.e., 0·0540, there would fall in the range so obtained values which would be the most frequently occurring means and would be such as would lie within the permissible range of random variation. The permissible range obtained from mode ± 0·0540 is from 7·2378 to 7·3458. It is seen from the observed data that out of 50 means only the following seven fall within this range :—

Serial number (*vide* Table II).	Actual mean diameter.
12	7·2916
14	7·2931
25	7·2846
26	7·2648
30	7·3126
35	7·2784
36	7·2775

These seven cases have, therefore, been taken for the calculation of standard values. The frequency distributions of these seven cases are set out in Table IV :—

TABLE IV.

Frequency distribution of the seven cases who fall in the homogeneous group.

Mean diameter.	SERIAL NUMBER OF CASES.						
	26	36	35	25	12	14	30
4·50	..	..	..	..	..	..	..
4·75	..	..	..	..	..	..	..
5·00	1	..	..	..	..	1	..
5·25	..	..	..	..	..	..	..
5·50	..	..	..	..	1	2	..
5·75	1	1	1	..	..	..	1
6·00	3	2	1	4	1	8	2
6·25	11	2	7	6	4	7	8
6·50	28	19	20	19	21	15	18
6·75	55	70	52	40	43	51	45
7·00	108	113	111	125	119	100	112
7·25	121	127	114	127	131	113	113
7·50	99	119	93	106	118	80	89
7·75	66	77	59	61	54	74	55
8·00	24	17	20	19	27	35	30
8·25	20	11	16	10	8	8	14
8·50	5	2	6	6	8	6	11
8·75	..	3	1	4	1	5	5
9·00	..	..	..	..	..	..	..
9·25	..	..	..	..	..	..	..
9·50	..	..	..	..	..	..	..
TOTAL FREQUENCY	542	563	501	527	536	505	503

Analysis of variance on the data relating to these seven persons is shown below :—

TABLE V.

Analysis of variance based on seven cases, viz., numbers 12, 14, 25, 26, 30, 35, and 36.

Source of variance.	Degrees of freedom.	Sum of squares.	Mean variance.
Between persons ..	6	0·7124	0·1187
Residual ..	3,670	768·4598	0·2094
TOTAL ..	3,676	..	..

The variation from ' person to person ' may therefore be taken to be of the same order as the ' residual '.

The mean value obtained from these seven cases   = 7·286
Estimate of standard deviation from the residual = 0·458
The standard error of the mean of 500 cells   = 0·02046

If 1 per cent level of significance is taken we may say that because of errors of random sampling the variation in the standard mean will be ± 2·576 × 0·02046, i.e., ± 0·0527.   In other words, means lying within the range 7·233 to 7·339 belong to healthy persons.

As suggested by Price-Jones (*loc. cit.*) two normal curves have been calculated on the basis of these two extreme values and are presented in Table V.   These provide the frequencies with which cell diameters may be found to occur in the two extreme cases.

TABLE VI.

Showing the frequency distribution expected when normal curve is fitted to extreme mean values 7·233 and 7·339, the standard deviation being 0·458.

Class intervals.	EXPECTED DISTRIBUTIONS.	
	Mean = 7·233.	Mean = 7·339.
5·75	1	1
6·00	6	4
6·25	20	12
6·50	46	33
6·75	80	65
7·00	107	97
7·25	100	107
7·50	76	89
7·75	41	55
8·00	17	26
8·25	5	9
8·50	1	2
TOTAL ..	500	500

It will be noticed that this treatment has provided a very narrow range within which means of the healthy persons may be expected to lie.   It may be asked : What is the practical utility of this range when it excludes 43 out of a sample of 50 persons ?   The answer to this can be that the above treatment does not indicate that means falling outside this range are to be regarded as belonging to unhealthy persons.   All that it shows is that the 50 means cannot be regarded as belonging to the same class or source, for were it so, it is certain that their range of variation would be of the same order of magnitude as calculated above.   Whether or not this is the usual finding could be ascertained by carrying out similar investigations in different populations or by examining similar data collected by other workers

by the same methods. From Price-Jones' book we have measurements of 500 cells in the case of each of 100 healthy persons.

An examination of his data shows that there also is evidence of heterogeneity. This is clear from an analysis of variance, Table VII, presented below :—

TABLE VII.

Analysis of variance of Price-Jones' data relating to 100 persons.

Source of variation.	Degrees of freedom.	Sum of squares.	Variance.	REMARKS.
Between persons ..	99	1,471·8106	14·8668	Significantly greater than residual.
Residual	49,900	10,413·9428	0·2087	..
TOTAL ..	49,999	11,885·7534	..	..

In order to secure homogeneity, therefore, as a first step, his data relating to age were used to work out a co-efficient of correlation between mean diameters and age. This correlation was found to be insignificant. The procedure further adopted has been the same as in connection with Indian figures. Price-Jones has given the frequency distribution of the 100 mean values. Pearsonian type I curve was fitted to this distribution and the following equation was obtained :—

$$Y = 11 \cdot 6677 \left[1 + \frac{X}{6 \cdot 3639} \right]^{2 \cdot 1215} \left[1 - \frac{X}{18 \cdot 9608} \right]^{6 \cdot 3208}$$

The modal value as determined from this equation is 7·26332. The standard error of the mean calculated from the residual variance is 0·02043. The permissible range of variation of the means due to random causes around the modal value may be taken to be ± 0·05263. In other words we may include within the range of chance variation mean values, lying between 7·21069 and 7·31595. It is seen that there fall in this range only 26 persons whose serial numbers are : 3, 4, 6, 12, 14, 16, 18, 20, 24, 25, 34, 43, 44, 46, 49, 52, 58, 63, 65, 70, 75, 78, 85, 96, and 97.

The analysis of variance on data relating to these 26 persons is set out in Table VIII :—

TABLE VIII.

Analysis of variance table.

Source of variation.	Degrees of freedom.	Sum of squares.	Mean variance.	REMARKS.
Between persons ..	25	12·9457	0·5178	Significantly greater than the residual.
Residual	12,974	2,667·4234	0·2056	..
TOTAL ..	12,999	2,680·3691	..	..

This is an unexpected result. On further investigation, it is seen that this significance may be ascribed to two reasons :—

(1)   A different estimate of mean for person No. 25 in Price-Jones' table. We have not been able to reconcile this difference.

(2)   An unusually larger clustering of values at the extremes.

In order to get data not showing heterogeneity we excluded one value from each tail end. The persons Nos. 6 and 52 were thus left out. The results of an analysis of variance carried out on data relating to 23 persons is presented below. Judged on 1 per cent level this set does not exhibit heterogeneity.

TABLE IX.

Analysis of variance of r.b.c. data relating to persons Nos. 3, 4, 12, 14, 15, 16, 18, 20, 24, 34, 43, 44, 46, 49, 58, 63, 65, 70, 75, 78, 85, 96, and 97.

Source of variation.	Degrees of freedom.	Sum of squares.	Mean variance.
Between persons ..	22	7·2164	0·3280
Residual	11,477	2,349·7480	0·2047
TOTAL ..	11,499	2,356·9644	..

It is of interest that in the case of Bengalee men homogeneity was obtained over 7 out of 50 persons (14 per cent) whereas in Price-Jones' data 23 per cent belong to a homogeneous set. The difference between the two proportions is, however, not different statistically.

DISCUSSION.

Purely from a consideration of the laws of probability the mean values must be regarded as belonging to a variety of sources, as far as their r.b.c. measurements are concerned. If, and only if, it can be contended that

(*i*)   there may be certain factors which, though significantly affecting r.b.c. diameters, may not produce appreciable difference in a person's health,

(*ii*)   that the factors which may materially affect health may be of such a nature as to produce significantly greater variation than that produced by factors falling under (*i*), then we may adopt a wider range for our selection.

In that case we will perhaps have to regard these 50 persons as providing us the only available estimate of the errors due to minor causes mentioned under (*i*). The estimate of their variation can be calculated from the mean

variance corresponding to 'between persons' as given in Table I.   If we denote by $\sigma_{\overline{m}}$ the standard error of means and by $\sigma_{\overline{r}}$ the residual standard deviation then $\sqrt{\sigma_{\overline{m}}^2 + \sigma_{\overline{r}}^2/500}$ will measure the variation expected in the means due to a combination of random as well as of those causes which though significantly greater than the random are to be considered of minor importance in comparison with the factors under study.   The value $\sqrt{\dfrac{\sigma_{\overline{m}}^2 + \sigma_{\overline{r}}^2}{500}}$ can then be obtained by dividing the 'between persons' variance by 500, and taking the square root. The value thus obtained for the error of the mean is 0·250.   We may therefore say that $\pm\, 0\cdot250 \times 2\cdot576$, i.e., 0·644, can perhaps be taken as the permissible range of variation due to factors (i) and random causes.   The mean value of r.b.c. diameters for 50 persons is 7·288.   The extreme values therefore are 6·644 and 7·932.   The ideal curves obtained for these extreme values when the standard deviation is 0·468 are given in Table X.   It may be mentioned that 0·468 is the estimate of the standard deviation obtained only from the residual variances of the 50 frequency distributions.

TABLE X.

Showing the normal frequency distributions expected for the smallest and the largest mean r.b.c. diameter for healthy Bengalees.

Class intervals.	FREQUENCIES.	
	Largest mean diameter = 7·932. Standard deviation = 0·468.	Smallest mean diameter = 6·644. Standard deviation = 0·468.
5·00–5·25	..	1
5·25–5·50	..	3
5·50–5·75	..	10
5·75–6·00	..	28
6·00–6·25	..	58
6·25–6·50	1	89
6·50–6·75	2	105
6·75–7·00	9	94
7·00–7·25	25	63
7·25–7·50	53	32
7·50–7·75	85	12
7·75–8·00	105	4
8·00–8·25	97	1
8·25–8·50	68	..
8·50–8·75	36	..
8·75–9·00	14	..
9·00–9·25	4	..
9·25–9·50	1	..
	500	500

The frequencies can be represented by the two curves shown in the Graph :—

GRAPH.

PRICE-JONES' CURVE.

From Bengalee population.
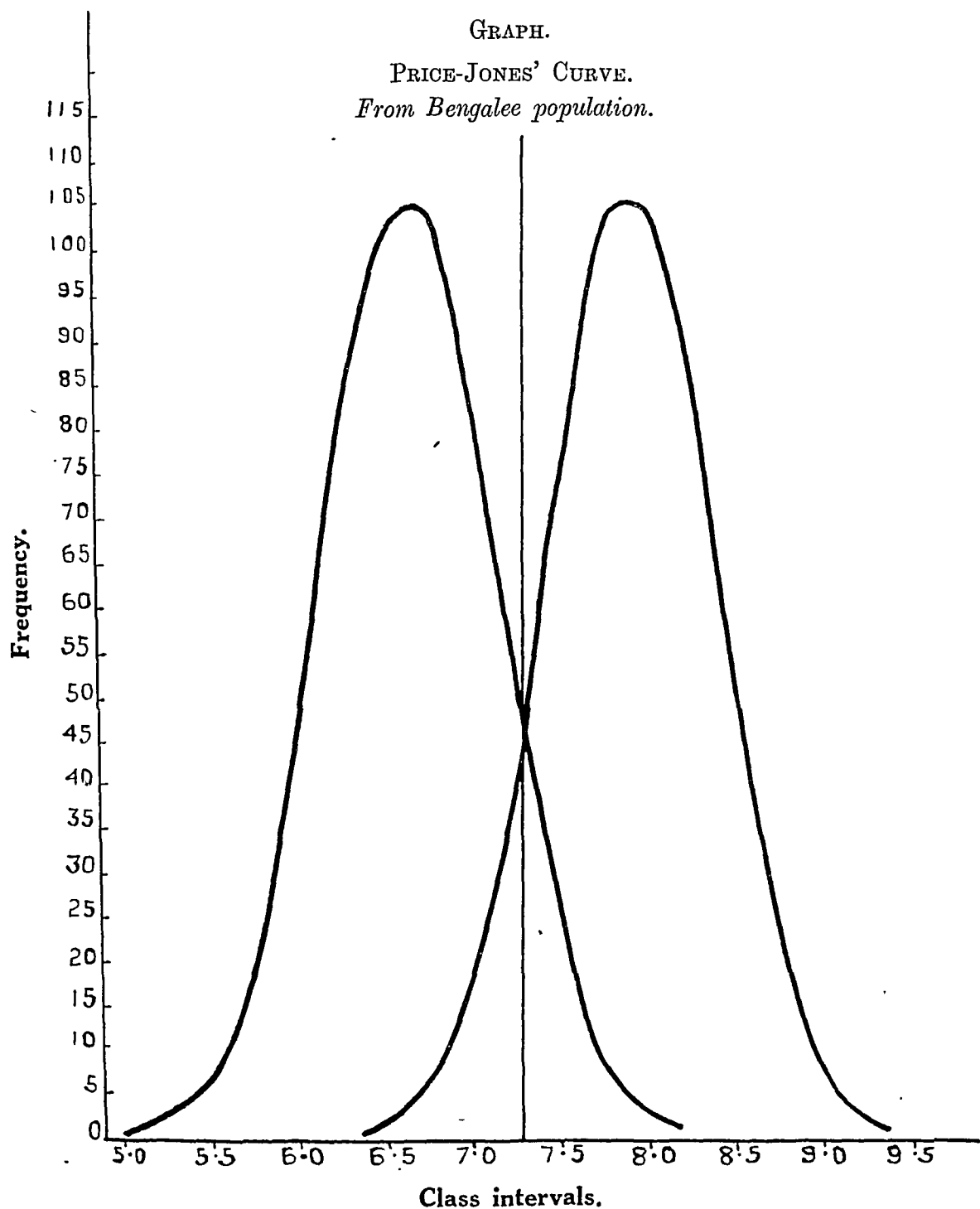
Normal frequency curves corresponding to the largest and the smallest mean red-blood-cell diameters for healthy Bengalees.

The limitations of this result should be carefully noticed. We have postulated that there are two types of factors, viz., (*a*) minor and (*b*) major, and that for the purpose of the present study, the minor factors, although significantly greater than the random causes, are to be considered of negligible importance. In actual fact it may not be possible to draw a line of demarcation between the minor and major factors because the difference may be a question of degree. For the purpose of this study however we have tacitly assumed that on the 50 persons no major factors operated.

It will be observed that we have not allowed the same range of variability as adopted by Price-Jones. His choice of three times the standard deviation, which represents a significance level of 1 in 369 against our figure of 2·576 times which represents a significant level of 1 in 100, was an arbitrary one. The greater heterogeneity of the data from our so-called normal individuals seems to justify our adoption of a lower level of significance in calculating ideal distributions for practical use.

Conclusion.

The mean of the mean diameters of 500 red cells of each of 50 different individual healthy male Bengalee Indians was 7·288 μ against Price-Jones' mean of 7·202 μ. To obtain the ideal distributions for the smallest and largest, respectively, mean diameters within normal limits, we have based our calculations on a 1 per cent level of significance. By this means we have obtained distributions with means of 6·644 μ and 7·932 μ, against Price-Jones' figures of 6·686 and 7·718 μ, respectively.

We suggest that our two distributions should be used in place of Price-Jones' distributions for Bengalee populations, and, until such time as standard distributions have been worked out for these, for other Indian populations living at sea-level.

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PRICE-JONES, C. (1933) ..   .. 'Red Blood Cell Diameters.' Oxford University Press, London.
SANKARAN, G., and RAO, M. V.   *Ind. Jour. Med. Res.*, **25,** p. 951.
RADHAKRISHNA (1938).

Frequency distribution of r.b.c. diameter on each of the 50 normal healthy persons.

Serial number:—	1	2	3	4	5	6	7	8	9	10	11	12	13
Name:—	S. C. B.	B. M.	B. G.	D. N. M.	G. M.	B. G.	S. N. C.	P. G. B.	R. G.	H. C. C.	K. C. B.	P. B.	P. N. B.
r.b.c. diameter.													
4·50	..	..	..	..	..	..	..	..	..	1	..	..	..
4·75	..	..	..	..	..	..	..	..	..	0	..	..	..
5·00	..	..	..	..	..	..	..	..	..	2	1	..	..
5·25	..	..	..	..	..	..	..	..	..	0	0	..	..
5·50	..	..	..	1	..	..	..	2	..	5	3	1	..
Carried over ..	..	..	..	1	..	..	..	2	..	8	4	1	..

Serial number:	1	2	3	4	5	6	7	8	9	10	11	12	13
Name: / r.b.c. diameter.	S. C. B.	B. M.	B. G.	D. N. M.	G. M.	B. G.	S. N. C.	P. G. B.	R. G.	H. C. C.	K. C. B.	P. B.	P. N. B.
Brought forward ..	..	..	..	1	..	..	..	2	..	8	4	1	..
5·75	1	7	..	4	1	2	..	1	..	11	2	0	..
6·00	0	10	..	12	5	4	1	6	..	41	21	1	1
6·25	5	26	1	21	21	23	0	21	1	62	39	4	3
6·50	15	74	7	56	49	40	3	27	1	96	87	21	7
6·75	50	154	16	79	83	91	15	65	20	147	142	43	25
7·00	88	176	78	166	167	138	33	168	38	110	182	119	116
7·25	116	84	98	101	104	113	98	137	71	67	89	131	127
7·50	95	31	106	48	53	78	143	68	115	24	29	118	118
7·75	69	6	102	13	28	39	122	27	115	3	13	54	61
8·00	38	3	55	7	4	19	62	11	68	3	3	27	31

8·25	20	1	30	5	4	9	24	7	40	0	3	8	24
8·50	8	..	13	1	1	1	11	0	35	1	1	8	20
8·75	6	..	6	..	1	0	5	1	29	..	..	1	8
9·00	..	..	2	..	..	1	3	..	11	..	..	..	4
9·25	..	..	..	..	..	..	..	..	2	..	..	..	..
9·50	..	..	..	..	..	..	..	..	..	..	..	..	..
TOTAL ..	511	572	514	514	521	558	520	541	546	573	615	536	545
Mean ..	7·370	6·880	7·53	6·84	7·036	7·114	7·586	7·1	7·738	6·721	6·864	7·29	7·434
Standard deviation ..	0·479	0·370	0·455	0·391	0·407	0·457	0·408	0·413	0·42	0·462	0·415	0·424	0·487
Co-efficient of variation ..	6·494	5·03	6·042	5·71	5·78	6·42	5·37	5·82	5·51	6·85	6·04	5·81	6·55
Age ..	30	30	24	28	35	35	30	23	24	24	31	27	38
Height in inches ..	67	64	68	68	70	65	66	69	65	68	68	64	67
Weight in pounds ..	146	134	116	154	125	116	144	160	127	114	140	114	144

APPENDIX—*contd.*

Serial number:—	14	15	16	17	18	19	20	21	22	23	24	25	26
Name:—	J. K. B.	G. S. N.	A. S. S.	S. C. R.	A. C. D.	A. K. D.	P. B.	N. G.	R. N.	G. K. R.	S. P. B.	D. D. M.	A. K. B.
r.b.c. diameter.													
4·50	..	..	..	..	..	..	..	..	..	..	..	..	..
4·75	..	..	..	..	..	..	..	..	..	..	..	..	..
5·00	1	..	..	..	..	..	..	1	..	..	..	..	1
5·25	0	..	..	..	..	..	..	0	..	..	..	..	0
5·50	2	..	..	..	..	..	1	0	..	..	7	..	0
5·75	0	1	..	1	..	1	0	5	..	..	5	..	1
6·00	8	2	..	6	1	2	1	6	2	..	13	4	3
6·25	7	6	1	8	0	21	5	18	8	2	37	6	11

6·50	6·75	7·00	7·25	7·50	7·75	8·00	8·25	8·50	8·75	Carried over
28	55	108	121	99	66	24	20	5.	..	542
19	40	125	127	106	61	19	10	6	4	527
64	110	129	74	32	25	10	3	3	1	513
3	18	73	117	150	108	57	23	14	12	577
25	57	158	125	101	59	21	1	1	..	558
43	77	159	117	76	26	4	3	..	..	535
12	37	77	86	116	84	61	16	5	5	506
49	105	120	96	62	37	18	14	5	1	531
5	39	96	133	107	78	38	8	1	2	508
44	72	144	124	70	42	6	3	1	..	521
7	16	67	82	121	111	69	20	8	22	524
7	37	91	110	93	86	55	14	17	11	530
15	51	100	113	80	74	35	8	6	5	505

Serial number:—	14	15	16	17	18	19	20	21	22	23	24	25	26
Name:—	J. K. B.	G. S. N.	A.S.S.	S. C. R.	A. C. D.	A. K. D.	P. B.	N. G.	R.N.	G. K. R.	S.P.B.	D. D. M.	A. K. B.
r.b.c. diameter. Brought forward	505	530	524	521	508	531	506	535	558	577	513	527	542
9·00	..	5	3	..	..	1	1	..	..	1	1	..	..
9·25	..	0	..	..	..	..	..	..	..	..	..	..	..
9·50	..	1	..	..	..	..	..	..	..	..	..	..	..
TOTAL	505	536	527	521	508	532	507	535	558	578	514	527	542
Mean	7·293	7·462	7·58	7·107	7·364	7·11	7·434	7·03	7·206	7·532	6·919	7·285	7·267
Standard deviation	0·505	0·529	0·478	0·375	0·385	0·490	0·474	0·430	0·387	0·437	0·507	0·440	0·45
Co-efficient of variation	6·92	7·08	6·30	5·27	5·22	6·90	6·38	6·11	5·37	5·80	7·33	6·01	6·18
Age	28	25	25	33	27	28	21	24	23	28	29	36	25
Height in inches	65	69	65	66	66	64	68	68	64	63	66	64	70
Weight in pounds	110	112	123	136	113	120	114	105	110	138	154	160	167

Serial number:—	27	28	29	30	31	32	33	34	35	36	37	38	39
Name:— (r.b.c. diameter)	P. C.	U. N. C.	N. N. D.	S. C. S.	H. M. D.	P. K. C.	K. B.	S. C. G.	D. O. C.	S. K. C.	M. A.	H. R.	A. T. D.
4·50	..	..	..	..	..	..	..	..	..	..	..	..	..
4·75	..	..	..	..	..	..	..	..	..	..	..	..	..
5·00	..	..	..	..	..	..	..	..	..	..	..	..	..
5·25	..	..	..	..	..	2	..	..	..	..	..	..	..
5·50	..	..	1	..	..	7	..	..	..	..	..	..	1
5·75	..	..	2	1	1	10	..	..	1	1	..	..	1
6·00	3	6	8	2	6	22	..	1	1	2	1	1	6
6·25	13	17	17	8	18	52	1	3	7	2	1	1	18
6·50	30	66	62	18	65	112	8	1	20	19	11	3	20
6·75	62	133	96	45	127	130	36	14	52	70	36	15	63
Carried over ..	108	222	186	74	217	335	45	19	81	94	49	20	109

APPENDIX—contd.

Serial number:—	27	28	29	30	31	32	33	34	35	36	37	38	39
Name:— r.bc. diameter.	P. C.	U. N. C.	N. N. D.	S. C. S.	H. M. D.	P. K. C.	K. B.	S. C. C. G.	D. O. C.	S. K. C.	M. A.	H. R.	A. T. D.
Brought forward	108	222	186	74	217	335	45	19	81	94	49	20	109
7·00	116	143	136	112	128	90	92	46	111	113	97	51	110
7·25	110	85	89	113	93	42	125	89	114	127	121	102	120
7·50	92	60	57	89	51	31	121	105	93	119	126	117	94
7·75	58	14	25	55	20	11	90	93	59	77	64	103	59
8·00	25	8	7	30	7	4	46	88	20	17	26	61	17
8·25	8	2	4	14	1	2	20	40	16	11	15	34	5
8·50	2	..	1	11	0	..	8	18	6	2	4	11	3
8·75	..	..	1	5	1	..	6	9	1	3	2	7	3

	Total													
9·00	..		1	1			2	3						
9·25	..		0											
9·50	..		1											
TOTAL	..	520	508	505	563	501	509	556	515	518	503	506	534	519
Mean	..	7·205	7·573	7·46	7·28	7·249	7·633	7·451	6·738	7·079	7·313	7·010	7·076	7·216
Standard deviation	..	0·464	0·445	0·411	0·406	0·442	0·465	0·447	0·469	0·376	0·485	0·438	0·386	0·444
Co-efficient of variation	..	6·439	5·876	5·511	5·577	6·11	6·08	5·99	6·90	5·30	6·60	6·20	5·44	6·14
Age	..	53	22	29	20	26	28	28	25	22	24	19	28	26
Height in inches	..	66	67	69	64	64	66	54	65	66	64	63	66	68
Weight in pounds	..	128	119	154	105	110	112	133	143	129	104	120	132	132

APPENDIX—contd.

Serial number:—	40	41	42	43	44	45	46	47	48	49	50
Name:—	S. N. B.	D. N. D.	G. G. D.	N. C. G.	L. M. B.	K. D. B.	S. C. D.	L. K. P.	H. K. M.	N. S.	S. B.
r.b.c. diameter.											
4·50	:	:	:	:	:	:	:	:	:	:	:
4·75	:	:	:	:	:	:	:	:	:	:	:
5·00	:	:	:	:	:	:	:	:	:	:	:
5·25	:	:	:	:	:	:	:	:	:	:	:
5·50	:	:	:	5	:	:	:	:	:	:	:
5·75	1	:	:	8	:	:	:	:	:	:	:
6·00	1	1	:	37	1	:	:	:	:	3	:
6·25	4	0	:	29	2	2	4	:	5	10	:

6·50	2	25	10	3	9	7	1	89	1	4	14
6·75	30	43	38	12	28	21	26	136	4	12	32
7·00	85	89	78	52	64	72	56	114	11	25	68
7·25	117	96	118	93	128	100	112	75	33	78	93
7·50	131	110	113	131	130	108	129	24	85	117	116
7·75	79	72	88	97	116	92	116	12	98	149	102
8·00	32	40	50	69	50	59	43	2	117	62	63
8·25	23	17	26	47	27	39	19	..	87	40	24
8·50	4	7	5	10	13	19	8	..	49	18	8
8·75	3	4	3	7	6	10	5	..	30	8	4
Carried over	506	516	534	521	575	529	518	531	515	514	530

Serial number:—	40	41	42	43	44	45	46	47	48	49	50
Name:—	S. N. B.	D. N. D.	G. G. D.	N. C. G.	L. M. B.	K. D. B.	S. C. D.	L. K. P.	H. K. M.	N. S.	S. B.
r.b.c. diameter. Brought forward	530	514	515	531	518	529	575	521	534	516	506
9·00	..	..	23	..	..	1	1	0	..	2	..
9·25	..	..	3	..	..	..	1	1	..	0	..
9·50	..	..	1	..	..	..	..	..	..	1	..
Total ..	530	514	542	531	518	530	577	522	534	519	506
Mean ..	7·47	7·655	7·978	6·79	7·495	7·553	7·498	7·603	7·072	7·36	7·43
Standard deviation ..	0·47	0·417	0·489	0·441	0·41	0·484	0·453	0·43	0·445	0·51	0·396
Co-efficient of variation ..	6·291	5·45	6·13	6·48	5·47	6·401	6·04	5·65	6·29	6·929	5·32
Age ..	26	33	20	32	27	30	30	18	30	41	28
Height in inches ..	65	64	64	71	63	70	65	62	66	67	67
Weight in pounds ..	154	112	99	140	123	132	100	100	113	127	117

THE USE OF DOGS FOR THE STANDARDIZATION OF DIGITALIS.

BY

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AND

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IF convenience and economy be the criteria for the selection of animals for the standardization of digitalis, the dog should be the animal of choice in this country. There is a strong sentiment against the killing of cats, and these animals are not readily available. But dogs are only too plentiful and it will certainly be an advantage if dogs could be substituted for cats in the bio-assay of digitalis.

Richaud (1914) in a general survey of assay methods for the cardiotonic group of drugs found that the dog generally reacted to intravenous administration, but the intensity of the cardio-vascular changes was quite variable and bore no relation to the weight of the dog or the quantity of the drug given, and also that death was caused by respiratory failure while the heart was still working. He, therefore, concluded that dogs were unsuitable for the determination of the therapeutic value of the cardiac tonics. Rowe (1919) tested a number of samples of ouabain and digitalis on cats and dogs and concluded that cats were less satisfactory than dogs. Haskell *et al.* (1928) used dogs under morphine and ether anæsthesia, injecting 1 in 10 dilution of tincture of digitalis into the vein at the rate of 2 c.c. every $2\frac{1}{2}$ minutes. No artificial respiration was used. The authors concluded that, while dogs were inferior to cats for the assay of digitalis, a fairly satisfactory assay may be carried out by using from six to ten dogs for each test. On the average dogs required 1·75 times as large a dose to kill as did cats. Tiffeneau, Levy and Pichot (1929) made an intensive study of the use of dogs for this assay under chloralose anæsthesia and artificial respiration. Experiments on ouabain showed that dogs irrespective of breed or origin possessed uniform sensitivity. In a subsequent communication, Levy and Pichot (1929) came to the conclusion that the Hatcher-Magnus method developed for cats could be used in the assay of digitalis upon dogs with a maximum error not exceeding 18 per cent. McGuigan and McGuigan (1938) used dogs anæsthetized with pentobarbital (35 mg. per kilo in 5 per cent solution intraperitoneally). Artificial respiration was not used in every case. Tincture of digitalis was injected into the femoral vein in a dose of 0·1 c.c. per kilo of body-weight every five minutes, at least one minute being taken for each injection. Their conclusion is that any one dog gives results within 20 per cent of the actual strength of the

drug ; any two will give results within 10 per cent.   As judged from the variations found with cats and frogs, dogs are quite as suitable for the standardization of digitalis.   The average time of an experiment was twelve injections which occupied exactly one hour.

<div align="center">OUR METHOD.</div>

We used dogs weighing from 4 to 7 kilograms, a few being puppies between 3 and 4 kilograms.   They were all anæsthetized by an intraperitoneal injection of chloretone in olive oil (0·2 g. per kilo), preceded by an injection of about 3 to 4 milligrams of morphine hydrochloride per kilogram of body-weight.   Cats were anæsthetized by an intraperitoneal injection of chloretone in alcohol (0·2 g. per kilo). It has been pointed out by several workers that, provided comparisons are made under identical conditions, the anæsthetic used was immaterial (David and Raja-manickam, 1934).   Spinal animals are perhaps the best as admitted by Burn.   We have in this series used chloretone as it is very easy to administer, the anæsthesia is quickly attained and is uniform both in cats and dogs.   Moreover, we find that neither urethane nor chloralose is suitable for the dog as reflex excitement occurs invariably in the early stages.   The method for assay was as described by de Lind van Wijngaarden mentioned by Burn (1928).   Artificial respiration was used in every case.   The tincture diluted 1 in 20 was slowly run into the saphenous vein and the m. l. d. required to bring the blood pressure to zero was calculated per kilogram weight for each animal.   The standard used by us was a tincture made from the International leaf powder (1928)* of which 0·1 g. was equal to one unit of activity. The results are given below.   Four unknown tinctures were assayed both by the cat method and on dogs.   The duration of each experiment was from 35 to 70 minutes.

<div align="center">TABLE I.</div>

<div align="center">*Standard tincture, diluted 1 in 20.*</div>

CAT.		DOG.	
Weight in kilograms.	m. l. d. per kg. in c.c.	Weight in kilograms.	m. l. d. per kg. in c.c.
2·5	19·00	5·4	29·40
3·9	18·20	4·4	42·95
2·6	17·69	3·8	22·89
2·5	20·00	6·5	27·54
2·9	13·11	4·4	19·77
1·4	16·78	4·8	27·39
2·0	15·50	4·1	23·17
3·2	16·87	2·9	20·69
1·5	19·33	3·5	28·00
2·5	17·60	3·4	28·38
2·3	16·95	4·8	25·31
2·2 .	18·18	3·3	28·03
1·9	16·58	4·0	25·00
AVERAGE ..	17·37	..	26·81

* Kindly supplied by the Biochemical Standardization Laboratory of the Government of India at Calcutta.

TABLE II.

Sample A, diluted 1 in 20.

CAT.		DOG.	
Weight in kilograms.	m. l. d. per kg. in c.c.	Weight in kilograms.	m. l. d. per kg. in c.c.
2·0	17·25	3·2	40·94
2·4	19·58	3·6	34·44
2·2	20·90	3·6	34·58
2·5	20·80	4·4	41·81
2·9	22·76	4·0	32·75
..	..	4·1	30·00
..	..	8·0	30·88
AVERAGE ..	20·26	..	35·06
Strength ..	85·73 per cent	..	76·46 per cent.
Difference in potency between cat and dog methods.		10·27 per cent.	

Sample A was a tincture manufactured on 7th July, 1937, with a potency certificate and obtained from the Medical Store Depôt, Madras, and kept unopened in the laboratory since then.

TABLE III.

Sample B, diluted 1 in 20.

CAT.		DOG.	
Weight in kilograms.	m. l. d. per kg. in c.c.	Weight in kilograms.	m. l. d. per kg. in c.c.
2·3	26·95	5·2	52·69
2·8	29·28	4·4	55·55
2·2	26·81	4·0	44·25
2·9	31·38	4·2	79·76
2·6	31·15	5·6	47·67
2·2	29·32	4·2	47·62
..	..	6·8	43·68
AVERAGE ..	29·15	..	55·98
Strength ..	59·58 per cent	..	50·55 per cent.
Difference in potency between cat and dog methods.		9·03 per cent.	

Sample B was a tincture manufactured on 2nd June, 1936, with a potency certificate, but with a rider to say that it should not be used after 2nd June, 1937. The bottle had been opened and partly used.

TABLE IV.

Sample C, diluted 1 in 20.

CAT.		DOG.	
Weight in kilograms.	m. l. d. per kg. in c.c.	Weight in kilograms.	m. l. d. per kg. in c.c.
2·0	24·00	3·9	32·82
2·5	26·40	3·7	32·48
3·1	22·26	5·0	34·00
2·9	17·58	4·5	28·22
2·5	24·00	3·3	45·78
AVERAGE ..	22·85	..	34·66
Strength ..	76·01 per cent	..	77·35 per cent.

Difference in potency between cat and dog methods.	1·34 per cent.

Sample C was a freshly prepared tincture made by a local firm. (Date of manufacture, presumably latter part of 1938.)

TABLE V.

Sample D, diluted 1 in 20.

CAT.		DOG.	
Weight in kilograms.	m. l. d. per kg. in c.c.	Weight in kilograms.	m. l. d. per kg. in c.c.
2·6	16·11	5·45	22·58
2·3	13·91	3·9	26·15
2·2	16·36	4·2	27·50
2·6	17·11	7·3	25·20
1·85	14·86	6·2	24·51
AVERAGE ..	15·67	..	25·19
Strength ..	110·85 per cent.	..	106·43 per cent.

Difference in potency between cat and dog methods.	4·42 per cent.

Sample D was bought locally and was manufactured on 4th November, 1938, by a reputable firm in this country.

From the above data it is seen that the dog usually gives a lower figure as compared to the standard, but the error is within experimental limits and anyway not more than 10 per cent. In sample C, the error was 1·34 per cent, practically negligible. In the case of sample D which showed a potency about equal to the standard, the difference of 4·42 per cent is also well within the limits of experimental error, and therefore inappreciable. Generally speaking the m. l. d. for the dog is higher per kilogram weight, the ratio being about 1 : 1·68, *vide* Haskell *et al.* (*loc. cit.*) who obtained a ratio of 1 : 1·75. The individual variation in the m. l. d. when a number of dogs are used is not more than is found with cats.

CONCLUSIONS.

We are, therefore, of opinion that dogs can very well replace cats for the bio-assay of digitalis without any sacrifice of accuracy of results ; and dogs are certainly cheaper, easier to get, and more convenient to handle than cats.

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The following has been received for publication :—

ANNOUNCEMENT

Of the Francis Amory Septennial Prize of the American Academy of Arts and Sciences under the Will of Francis Amory.

IN compliance with the requirements of a gift under the will of the late Francis Amory of Beverly, Massachusetts, the American Academy of Arts and Sciences announces the offer of a septennial prize for outstanding work with reference to the alleviation or cure of diseases affecting the human genital organs, to be known as the Francis Amory Septennial Prize. The gift provides a fund, the income of which may be awarded for conspicuously meritorious contributions to the field of knowledge ' during the said septennial period next preceding any award thereof, through experiment, study or otherwise . . . . in the diseases of the human sexual generative organs in general '. The prize may be awarded to any individual or individuals for work of ' extraordinary or exceptional merit ' in this field.

In case there is work of a quality to warrant it, the first award will be made in 1940. The total amount of the award will exceed ten thousand dollars, and may be given in one or more awards. It rests solely within the discretion of the Academy whether an award shall be made at the end of any given seven-year period, and also whether on any occasion the prize shall be awarded to more than a single individual.

While there will be no formal nominations, and no formal essays or treatises will be required, the Committee invites suggestions, which should be made to the Amory Fund Committee, care of the American Academy of Arts and Sciences, 28 Newbury Street, Boston, Massachusetts, U. S. A.

—Editor,
Indian Journal of Medical Research.

OBSERVATIONS ON THE 'H' ANTIGEN OF VIBRIOS.

BY

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CORRIGENDUM.

In paper entitled 'Serological Reactions in Kala-azar: Complement-Fixation, etc.' by S. D. S. Greval, P. C. Sen Gupta and L. E. Napier, *Ind. Jour. Med. Res.,* **27,** 1, July, 1939, on pages 184 and 185, in lines 8 and 9 respectively, *read* ' 137 cases ' in place of ' 132 cases '.

—ED.

than those corresponding to that of *V. cholerœ.* The present communication deals with a study directed towards ascertaining—

(a) What proportion of inagglutinable vibrios in a varied collection from sources such as cases of clinical cholera, healthy individuals, and water, show 'H' relationship with the agglutinable *V. cholerœ.*

(b) Whether the flagellar antigen of vibrios showing 'H' agglutination with cholera high-titre serum but not 'O' agglutination is partially or completely identical with that of *V. cholerœ.*

(c) Whether a common 'H' antigen exists amongst vibrios which do not show 'H' serological relationship with *V. cholerœ.*

(a) Two hundred and nineteen strains of vibrios which were not agglutinated by group I (G and V) 'O' serum, comprising 56 strains isolated from cholera cases, 35 strains from healthy persons and 128 strains from water, have been examined. The serological tests were chiefly made with 'H' + 'O' serum prepared against the classical Inaba cholera strain and the test suspensions used

were (1) formalized agar washings of an opacity of 2,000 million vibrios per c.c. and (2) chloroformed extracts of 4,000 million vibrios per c.c. prepared according to the method of Vassiliadis (1937). The distribution of the strains according to their sugar reactions (Heiberg classification) and their 'H' relationship to *V. choleræ* are given in Tables I and II :—

TABLE I.

Formalized agar washings 2,000 million vibrios per c.c. used as test suspensions.

Heiberg types.	NAG VIBRIOS.								
	CASE STRAINS.			STRAINS FROM HEALTHY INDIVIDUALS.			WATER STRAINS.		
	Number of strains tested.	'H' positive.	'H' negative.	Number of strains tested.	'H' positive.	'H' negative.	Number of strains tested.	'H' positive.	'H' negative.
I M + S + A −	8	2	6	6	2	4	34	19	15
II M − S + A −	5	4	1	6	5	1	48	39	9
III M + S + A +	27	5	22	18	3	15	7	0	7
IV M − S + A +	9	0	9	3	0	3	22	0	22
V M + S − A −	6	0	6	1	0	1	7	0	7
VI M − S − A −	1	0	1	1	0	1	10	0	10
TOTALS	56	11	45	35	10	25	128	58	70

'H' positive case strains   .. 19·6 per cent
'H' positive carrier strains   .. 28·6   ,,   } Average 35·5 per cent.
'H' positive water strains   .. 42·0   ,,
M = Mannose; S = Saccharose; A = Arabinose.
+ = Acid; − = No change.

TABLE II.

Chloroform extracts 4,000 million vibrios per c.c. used as test suspensions.

Heiberg types.	NAG VIBRIOS.								
	CASE STRAINS.			STRAINS FROM HEALTHY INDIVIDUALS.			WATER STRAINS.		
	Number of strains tested.	'H' positive.	'H' negative.	Number of strains tested.	'H' positive.	'H' negative.	Number of strains tested.	'H' positive.	'H' negative.
I M + S + A − ..	8	3	5	6	4	2	34	27	7
II M − S + A − ..	5	4	1	6	6	0	48	47	1
III M + S + A + ..	27	10	17	18	10	8	7	0	7
IV M − S + A + ..	9	0	9	3	0	3	22	0	22
V M + S − A − ..	6	6	0	1	0	1	7	3	4
VI M − S − A − ..	1	1	0	1	1	0	10	0	10
TOTALS ..	56	24	32	35	21	14	128	77	51

'H' positive case strains   .. 42·9 per cent
'H' positive carrier strains   .. 60   ,,   } Average 53 per cent.
'H' positive water strains   .. 56·5   ,,
M = Mannose; S = Saccharose; A = Arabinose.
+ = Acid; − = No change.

The facts that emerge from a consideration of the tables are :—

(1) Using formalized suspensions 35·5 per cent of strains examined show an 'H' antigen in common with *V. choleræ*. With chloroform extracts the percentage of 'H' positive strains is 53 per cent (42·9 per cent of case strains, 60 per cent of carrier strains, and 56·5 per cent of water strains reacting with a cholera 'H' + 'O' serum to 50 per cent or over of the titre of the serum).

By the use of ' H ' + ' O ' serum in diagnosis 35·5 per cent of non-cholera strains in this series would have been normally diagnosed as *V. choleræ* if this test alone had been relied on.

(2) The great majority of the ' H ' positive strains belong to Heiberg types I and II, while Heiberg type IV strains (34 strains tested) are all ' H ' negative.

(3) The highly sensitive nature of the chloroform extracts in demonstrating the flagellar antigen of vibrios is well brought out by the comparative tests carried out in this series. A larger number of strains become positive after such treatment and they show much higher titres.

(b) *Whether the flagellar antigen of ' H ' agglutinable but ' O ' inagglutinable non-cholera vibrios is partially or completely identical with that of the cholera vibrio.*

To eliminate the possibility of a common somatic antigen being present amongst the strains used in this study only such strains were selected as did not agglutinate with any one of the 31 special ' O ' sera raised against non-cholera vibrios isolated from cases, carriers, and waters and used by Taylor *et al.* (*loc. cit.*) in their classifications of Indian strains.

Agglutination and agglutinin absorption tests were carried out with pure ' H ' sera prepared by absorption of the sera raised against living suspensions of ten selected strains with massive doses of heat-killed cultures of the homologous vibrios.

Extracts of vibrios in 15 per cent chloroform were used as test suspensions. The value of such extracts for the demonstration of ' H ' antigen of vibrios is shown in the previous section.

The following strains were used in the tests :—

1. Vibrio Bunt—Calcutta case strain isolated on 18th June, 1936. Inagglutinable with group I ' O ' serum, Heiberg type II, non-hæmolytic.

2. Vibrio 2019—Mandapam carrier strain isolated in 1936. Inagglutinable with group I ' O ' serum, Heiberg type VI, non-hæmolytic.

3. Vibrio 361 1—Calcutta case strain isolated on 7th July, 1935. Inagglutinable with group I ' O ' serum, Heiberg type I, hæmolytic.

4. Vibrio 27—Water strain isolated on 14th October, 1936. Inagglutinable with group I ' O ' serum, Heiberg type V, hæmolytic.

5. Vibrio 9 E—Water strain isolated on 10th April, 1937. Inagglutinable with group I ' O ' serum, Heiberg type II, non-hæmolytic.

6. Vibrio G 7—Madras case strain isolated on 29th August, 1932. Inagglutinable with group I ' O ' serum, Heiberg type III, non-hæmolytic.

7. Vibrio 21—Mandapam carrier strain isolated on 23rd June, 1932. Inagglutinable with group I ' O ' serum, Heiberg type III, non-hæmolytic.

8. Vibrio 1801—Calcutta case strain isolated on 25th May, 1936. Inagglutinable with group I ' O ' serum. Heiberg type V, non-hæmolytic.

9. Vibrio 1970/1—Calcutta case strain isolated on 19th June, 1936. Inagglutinable with group I ' O ' serum. Heiberg type VI, hæmolytic.

10. Vibrio 1811/1—Calcutta case strain isolated on 28th May, 1936. Inagglutinable with group I ' O ' serum. Heiberg type I, hæmolytic.

The results are given in Tables III, IV, and V. It is shown that vibrios possessing the ' H ' antigen in common with *V. choleræ* can be divided into three main groups :—

(1) Strains showing complete identity of the ' H ' fractions of an inagglutinable non-cholera vibrio with *V. choleræ*.

(2) Strains possessing ' H ' antigen the major portion of which is identical with that of *V. choleræ* and a minor individual fraction in addition.

(3) Vibrios possessing a minor ' H ' fraction identical with that of *V. choleræ*. and a major individual fraction.

(c) Whether a common ' H ' antigen exists amongst vibrios which do · not show ' H ' serological relationship with V. choleræ.

Of 219 strains examined 97 were not agglutinated by Inaba ' H ' + ' O ' serum either in the formalized state or when chloroform extracts were used (Table II). High-titre sera were prepared against eight such strains representative ·of each of the Heiberg types and thirty strains inagglutinable with Inaba ' H ' + ·O ' serum were tested against each one of these sera to ascertain if a common ' H ' existed amongst the group not agglutinated by cholera high-titre serum.

' H ' + ' O ' sera raised against vibrios of Heiberg types I, Ia, V, and VI did not agglutinate any one of the 30 strains tested.

' H ' + ' O ' sera of Heiberg types IV and II vibrios agglutinated 1 and 2 strains respectively out of 30 strains examined.

' H ' + ' O ' serum prepared against a Heiberg type III vibrio agglutinated 7 out of 30 strains to 25 per cent of titre of the serum. This was confirmed by raising a high-titre serum against another strain belonging to Heiberg type III and 8 strains out of 30 tested were agglutinated by this serum. All these strains which showed a partial ' H ' relationship with the Heiberg type III vibrios belonged to Heiberg type III or IV.

It appears that vibrio strains not agglutinated by a cholera ' H ' + ' O ' serum form a heterogeneous group, some of them showing partial ' H ' relationship amongst themselves. This relationship is most manifest in the case of vibrios in the series tested which belong to Heiberg types III and IV.

TABLE III.

Showing that an 'H' agglutinable non-cholera vibrio can deplete the 'H' serum of an agglutinable cholera vibrio of all its flagellar antigen and vice versa.

Serum.	Absorbed with living suspension of	Absorbing dose.	Tested against	DILUTION.				TITRE OF UNABSORBED SERUM.		
				200	400	800	1,600 to 25,600.	Serum.	Tested against	Titre.
Inaba 'H' ..	9 E	$3{,}000 \times 10^9$	9 E	—	—	—	—	Inaba 'H'	Inaba	1/7,500
			Inaba	—	—	—	—	Inaba 'H'	9 E	1/7,500
9 E 'H' ..	Inaba	$3{,}600 \times 10^9$	9 E	+	—	—	—	9 E 'H'	9 E	1/7,500
			Inaba	—	—	—	—	9 E 'H'	Inaba	1/7,500
Inaba 'H' ..	Bunt	$3{,}000 \times 10^9$	Bunt	+	trace	—	—	Inaba 'H'	Inaba	1/12,800
			Inaba	—	—	—	—	Inaba 'H'	Bunt	1/12,800
Bunt 'H' ..	Inaba	$3{,}600 \times 10^9$	Bunt	—	—	—	—	Bunt 'H'	Bunt	1/6,400
			Inaba	—	—	—	—	Bunt 'H'	Inaba	1/3,200
Inaba 'H' ..	2019	$3{,}600 \times 10^9$	2019	—	—	—	—	Inaba 'H'	Inaba	1/12,800
			Inaba	—	—	—	—	Inaba 'H'	2019	1/12,800
2019 'H' ..	Inaba	$3{,}600 \times 10^9$	2019	—	—	—	—	2019 'H'	Inaba	1/3,200
			Inaba	—	—	—	—	2019 'H'	2019	1/3,200

The flagellar antigen of these three strains—9 E, Bunt and 2019—shows a complete identity with the 'H' antigen of cholera vibrio Inaba.

TABLE IV.

Vibrios possessing a major 'H' fraction identical with 'H' antigen of a cholera vibrio and in addition a minor individual 'H' component.

Serum.	Absorbed with living suspension of	Absorbing dose.	Tested against	DILUTION.				TITRE OF UNABSORBED SERUM.		
				200	400	800	1,600 to 25,600	Serum.	Tested against	Titre.
Inaba 'H' ..	361/1	3,600 × 10⁹	361/1	++	++	—	—	Inaba 'H'	Inaba	1/12,800
			Inaba	+	—	—	—	Inaba 'H'	361/1	1/12,800
361/1 'H' ..	Inaba	3,600 × 10⁹	361/1	++++	++++	++++	—	361/1 'H'	361/1	1/6,400
			Inaba	+	—	—	—	361/1 'H'	Inaba	1/3,200
Inaba 'H' ..	1811/1	2,400 × 10⁹	1811/1	—	—	—	—	Inaba 'H'	Inaba	1/6,400
			Inaba	—	—	—	—	Inaba 'H'	1811/1	1/6,400
1811/1 'H' ..	Inaba	2,400 × 10⁹	1811/1	++++	++++	++	—	1811/1 'H'	1811/1	1/6,400
			Inaba	+	—	—	—	1811/1 'H'	Inaba	1/6,400

TABLE V.

Vibrios showing a small 'H' fraction in common with 'H' of a cholera vibrio but in addition having a major individual 'H' fraction.

Serum.	Absorbed with living suspension of	Absorbing dose.	Tested against	200	400	800	1,600	3,200	6,400	12,800	Serum.	Tested against	Titre.
Inaba 'H'	G. 7	1,000 × 10⁹	G. 7	—	—	—	—	—	—	—	Inaba 'H'	Inaba	3,200
			Inaba	++++	++++	++++	++++	++++	—	—	Inaba 'H'	G. 7	1,600
G. 7 'H'	Inaba	1,000 × 10⁹	G. 7	++++	++++	++	++	++	+	—	G. 7 'H'	Inaba	400
			Inaba	—	—	—	—	—	—	—	G. 7 'H'	G. 7	6,400
Inaba 'H'	Cm 21	1,000 × 10⁹	Cm 21	—	—	—	—	—	—	—	Inaba 'H'	Inaba	3,200
			Inaba	++++	++++	++++	++++	++++	—	—	Inaba 'H'	Cm 21	1,600
Cm 21 'H'	Inaba	1,000 × 10⁹	Cm 21	+++	++	++	+	—	—	—	Cm 21 'H'	Cm 21	1,600
			Inaba	—	—	—	—	—	—	—	Cm 21 'H'	Inaba	100 mg.

Dilution.
Titre of unabsorbed serum.

A diversity in ' H ' antigens is shown similar to that which has been demonstrated in the case of ' O ' antigens.

SUMMARY.

1. Serological examination of a series of 219 vibrio strains inagglutinable by group I ' O ' serum (G and V) and isolated from sources such as cases of clinical cholera, healthy individuals, and water has shown that 35·5 per cent of these strains possess ' H ' antigen partially or completely identical with ' H ' of *V. choleræ*.

2. Cross-absorption tests carried out with pure ' H ' sera prepared against 10 such strains show that vibrios agglutinable by ' H ' + ' O ' cholera serum can be divided into three main groups :—

(1) Strains possessing ' H ' antigen identical with that of *V. choleræ*.

(2) Strains possessing ' H ' antigen the major portion of which is identical with that of *V. choleræ* and a minor individual fraction in addition.

(3) Strains possessing a minor ' H ' fraction identical with that of *V. choleræ* and a major individual fraction.

3. Strains not agglutinated by a cholera ' H ' + ' O ' serum form a heterogeneous group some of them showing partial ' H ' relationship amongst themselves but the majority possess individual ' H ' antigens.

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A PRELIMINARY NOTE ON THE RELATIVE EFFICIENCY OF BISMUTH-SULPHITE MEDIUM AND PEPTONE-WATER ENRICHMENT IN THE ISOLATION OF *V. CHOLERÆ* FROM HUMAN AND OTHER SOURCES.

BY

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IT is a matter of experience that many cases admitted to hospitals in Calcutta as ' clinical cholera ' do not show the presence of the agglutinable vibrio in the stools in spite of careful search and also that in many cases, where the isolations are effected by the use of alkaline peptone water, the proportion of agglutinable vibrios compared with *coliform* organisms and inagglutinable vibrios on the plates is small.

To overcome the above difficulties a modification of Wilson and Blair's (1931) medium was evolved by Read (1939), and simultaneously with the experimental studies of this new medium at the Central Research Institute, Kasauli, a preliminary field trial was instituted at Calcutta to compare its efficiency with that of alkaline peptone water at pH 9·2 as well as to ascertain whether under the conditions existing in the field the use of this medium would yield satisfactory isolation from natural waters and stools of carriers, where small concentrations of *V. choleræ* together with heavy opposing inocula may be expected to be present.

The earlier part of this work was carried out with Read's first modification of Wilson and Blair's liquid enrichment medium in which broth was replaced by peptone, mannose was substituted for mannite, brilliant green was omitted, and pH was adjusted at 9·2. During the rest of the period the second modification of the medium, in which the liq. bismuthi concentration was increased threefold, sodium chloride was replaced by sea-salt mixture and mannose was reduced to half, was

used with improved results. The composition of these media and mode of preparation have been described fully by Read (*loc. cit.*). A modification of Aronson's medium, in which sodium carbonate was reduced to $\frac{3}{4}$ths of the original strength and dextrin was completely omitted, was used to reduce the inhibitory effect of this medium on the growth of *V. choleræ* (vide *Appendix*). The following is a report of the results obtained during the course of the above investigations :—

A SHORT DESCRIPTION OF THE AREAS INVESTIGATED.

The investigation was carried out in three centres, viz., Rampurhat, Bashirhat, and Calcutta, the epidemiological features of these areas according to Lal (1938) being high epidemic, low epidemic, and endemic respectively. The Rampurhat area is situated about 150 miles from Calcutta and away from the deltaic tract, whereas the Bashirhat centre is in the deltaic region of South Bengal and is within forty miles from the city. Altogether 9 villages, 4 in Rampurhat and 5 in Bashirhat centres, were investigated. The villages in the latter area were further sub-divided into groups ' A ' and ' B ' according to their recent cholera history as given in Table I. The investigations in Calcutta were confined to the Cholera Ward, Campbell Hospital, for cases, and to the city and suburbs for water supplies.

SOURCES AND MATERIALS EXAMINED.

These are shown in Tables I-A and I-B. Altogether 658 samples consisting of 390 stools and 268 waters were examined. Of the stool samples 98 were collected from 51 cholera cases, 121 from 69 cholera contacts, and 171 from the same number of healthy individuals other than contacts. The water samples consisted of 242 from 117 tanks, 15 from 3 rivers, and 11 samples one each from 5 wells and 6 tube-wells. All samples of stool and water were examined in the modified Wilson and Blair's medium while 325 samples consisting of 203 stools and 122 waters were simultaneously examined by peptone-water enrichment. Ninety-eight of the stool samples examined in the latter medium were from cholera cases, 75 from contacts, and 30 from healthy non-contacts. Of the water samples 113 belong to tanks, 8 to rivers, and 1 only to tube-wells. The methods of collection and cultural technique have been described fully in the *Appendix*. It will be clear from this that one-half of the quantity of stools and less than half the quantity of waters only were examined in alkaline peptone as compared with the bismuth-sulphite medium.

RESULTS.

The results of isolation of *V. choleræ* from cases of cholera in the new bismuth-sulphite medium have been included in Table II for comparison with that of alkaline peptone water. It will be noted that though the figures are small they definitely indicate a greater success with the experimental medium than with alkaline peptone water, the percentages of positive isolations being 64·7 and 43·1 respectively. The difference is still more marked in view of the fact that of the 26 cases shown last in the table only three were clinically typical cholera cases.

Table III shows that 69 cholera contacts of which only 21 had a history of recent contact were examined. Four of the latter yielded *V. choleræ* simultaneously in the new medium and in alkaline peptone water. The old contacts*, on the other hand, were entirely negative in both the media. Of the 171 healthy non-contacts examined and shown in Table IV, none yielded *V. choleræ* in the new medium nor in alkaline peptone water in which only 30 of the total number of samples were simultaneously examined for the purpose of comparison. The results of examination of water samples from tanks, rivers, and wells have been shown in Table V. No *V. choleræ* was isolated from wells and rivers in spite of repeated examination of the latter sources. Eight of the 117 tanks, however, yielded *V. choleræ* with the new medium, whereas the corresponding positive isolations were only 5 with the alkaline peptone-water method ; moreover, out of 242 samples from these tanks the agglutinable vibrio was isolated from 10 by the former but only from 5 by the latter methods. Tables VI-A and VI-B show the comparative results of isolation of vibrios other than *V. choleræ* in the two media. On the elimination of this group of organisms partly depends the efficiency of the differential medium. It will, however, be found that 27·8 per cent of the samples examined in the new medium yielded inagglutinable vibrios as compared to 51·9 per cent in the alkaline peptone water. Of the former 12·6 per cent and of the latter 22·6 per cent were non-fermenters of mannose. The results thus indicate that compared with the alkaline peptone water there is some restriction (about 50 per cent) in the growth of the inagglutinable vibrios, particularly of the mannose non-fermenting types, in the new medium. The details of the Heiberg's groupings have been given in Table VI-A.

DISCUSSION.

In cases of clinical cholera in which the number of agglutinable vibrios may be expected to be relatively large, the above results show some difference in favour of the new medium. In the case of other human sources this difference was not established. The improved results in the examination of water sources were similar to those in cases of clinical cholera. It is probable, moreover, that the use of peptone water alkalinized to a pH of 9·2 in place of the more usual pH 8·0 to 8·5 may have improved the results obtained in alkaline peptone water. The bismuth-sulphite medium, however, was effective in reducing the number of inagglutinable vibrios present as well as *coliform* types, though the number of mannose-fermenting inagglutinable organisms was still very large. With regard to the failure to isolate *V. choleræ* from non-contacts the following points have to be considered : (1) In case of the Rampurhat specimens the stools were subjected to a delay of about 20 hours and in the case of Bashirhat 8 hours before examination, as the samples were collected 150 and 40 miles respectively from Calcutta. The water samples took two hours longer to allow for the filtration through the Seitz discs to be complete as far as practicable. (2) The cholera history of the areas as evident in Table I-A did not suggest that agglutinable vibrios would be commonly present in the stools of the normal population.

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* *Cf.* postscript at the end of the paper.

SUMMARY.

1.　A preliminary field trial was carried out in three centres in Bengal, viz., Rampurhat, Bashirhat, and Calcutta, to ascertain the comparative efficiency of Wilson and Blair's bismuth-sulphite fluid enrichment medium, as modified by Read, and alkaline peptone water at pH 9·2 for the isolation of *V. cholerae* from human and other natural sources.

2.　Altogether 658 samples consisting of 390 stools belonging to cases, contacts and healthy non-contacts and 268 water samples collected from tanks, rivers, wells, and tube-wells were examined.　While all of them were examined in the new medium only 325 samples, 203 of stools and 122 of water, were simultaneously examined in alkaline peptone water.

3.　Of the cases of clinical cholera 64·7 per cent yielded *V. cholerae* in the new medium as compared to 43·1 per cent in alkaline peptone water.　Only 4 of 21 recent contacts gave positive results with both media.　Eight of the 117 tank waters examined yielded positive isolations in the new media, whereas only 5 of them were positive in alkaline peptone water.　All other sources were negative.

4.　Compared with the alkaline peptone water there was some restriction of growth of the inagglutinable vibrios in the new medium and the majority (87·4 per cent) were mannose fermenters.

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　McV. (1931).

Postscript.—Of the 69 contacts referred to in this paper 48 gave an old history of contact with cholera cases. These, therefore, can be considered as good as healthy non-contacts.—[S. C. S.— 19-8-1939.]

APPENDIX.

TECHNIQUE.

Collection of materials.

In collecting samples of stools two teaspoonfuls, equivalent to 8 to 10 grammes, of the material were added to 20 c.c. of 2 per cent NaCl solution contained in 100-c.c. wide-mouthed sterile glass-stoppered bottles. Two to three drops of N/1 NaOH solution were then added to increase the alkalinity of the specimen. The quantity of salt solution was later increased to 40 c.c. with a proportional increase of the quantum of stool inoculum. After the replacement of sodium chloride by sea-salt mixture in the new selective medium stool samples were collected in sterile normal saline instead of in 2 per cent NaCl.

Samples of water were collected in sterile quart whisky bottles by dipping them directly into the source. To each of these bottles two teaspoonfuls of common salt were added to make the concentration between 1 and 2 per cent, each bottle accommodating about 750 c.c. of water when filled to the brim. For purposes of comparison water samples were also collected in 250-c.c. screw-capped medical bottles containing 20 c.c. of 10 per cent peptone and 10 per cent NaCl. This medium was directly inoculated with water at its source, the total volume being made up to 200 c.c. Eight drops of N/1 NaOH were then added to increase the alkalinity of the specimen.

Cultural technique.

On arrival of the specimens in the laboratory 10 c.c. of stools were examined as described by Read (loc. cit.) using the preliminary modification of Wilson and Blair's liquid enrichment medium for 458 specimens and final modification for 200 specimens. For purposes of comparison 2 c.c. of the emulsion were also inoculated into 20 c.c. of 1 per cent peptone water contained in the same kind of phial. In both cases the pH was brought up to 9·2 after inoculating the media with stools. The bottles were then incubated overnight at 37°C. and plated on agar and modified Aronson's* media. At least 10 and usually more likely colonies from each plate were picked off and examined in various ways for identification of the type or types of vibrios present. In using the final modification (Read's modification No. 2) of the bismuth-sulphite medium the pH invariably dropped and was re-adjusted 3 to 5 hours after the initial culturing, followed by a distinct improvement in the results of isolation. In case of the water samples collected directly in peptone water in the screw-capped phials the pH of the contents was adjusted to 9·2 in the same way as in stool culture and incubated directly. The salted samples were passed through

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*Aronson's medium was slightly modified to obtain better growth of vibrios. The plates were preserved in cold and in darkness wrapped up in blue paper. Those more than three days old lost much of their efficiency and were discarded. These should be used fresh as far as possible and therefore be made according to the requirements.

Seitz filters fitted with 6-cm. discs in quantities varying from 300 c.c. to 1,000 c.c. The discs were inoculated each into 20 c.c. of the selected medium freshly prepared in 100-c.c. wide-mouthed glass-stoppered bottles. The pH was adjusted to 9·2 as in other cases and the bottles incubated overnight at 37°C. Subsequent treatment was the same as in stool culture. In all cases the pH of the new medium was tested after overnight incubation. The very few cases where the reaction did not come down appreciably were incubated for a further period of 24 hours and plated again to study the colonies in the usual way.

TABLE I-A.

Showing the areas investigated and the number of samples examined from various sources.

Centre.	Epidemiological features.	Last outbreak.	Number of villages investigated.	Date of examination.	Cases. Number.	Cases. Samples.	Contacts. Number.	Contacts. Samples.	Non-contacts. Number.	Non-contacts. Samples.	Tanks. Number.	Tanks. Samples.	Rivers. Number.	Rivers. Samples.	Wells. Number.	Wells. Samples.	Tube-wells. Number.	Tube-wells. Samples.
...ampurhat	High epidemic	1936	4	July 1938 and December 1938.	..	..	12*	12	84	84	37	44	1	1	5	5	4	4
...shirhat Group A.	Low ,,	1931	3	August and September 1938.	..	..	36*	36	85	85	33	46	1	1	..	..	2	2
...shirhat Group B.	,, ,,	September 1938	2	September and October 1938.	3	19	21	73	2	2	8	28·	..	..	..	..	..	..
...lcutta ..	Endemic	..	..	May 1938 to March 1939.	48	79	..	..	..	..	39	124	1	13	..	..	..	..
				TOTALS ..	51	98	69	121	171	171	117	242	3	15	5	5	6	6

WATER-SUPPLIES.

* *Cf.* postscript at the end of the paper.

TABLE I-B.

Showing the number of sources and samples of stools and waters examined in two different media.

SOURCES EXAMINED.			SAMPLES EXAMINED.			STOOLS.			WATERS.			TOTAL NUMBER.	
Total number.	Stools.	Waters.	Total number.	Stools.	Waters.	Sources.	In W & B's medium.	In peptone water.	Sources.	In W & B's medium.	In peptone water.	Examined in W & B's medium.	Examined in peptono water.
422	291	131	658	390	268	Cases ..	98	98	Tanks	242	113	658	325
						Contacts ..	121	75	Rivers	15	8		
						Non-contacts	171	30	Wells	6	..		
						..	..	..	Tube-wells	5	1		
						TOTALS ..	390	203		268	122		

TABLE II.

Showing the comparative results of isolation of agglutinable vibrios in modified W & B's medium and alkaline peptone water from **cases.**

| Centre. | CASES. | | | | Date of examination. | Number of samples examined. | 'O' SUB-GROUP I POSITIVE. | | | Type of W & B's medium used. |
	Total number examined.	Total number of positive agg. vibrios.	In W & B's medium.	In 1 per cent peptone water.			Total number.	In W & B's medium.	In 1 per cent peptone water.	
Bashirhat Group B.	3	3	3	3	September 1938	11	7	7	6	M (1)
Campbell Hospital.	22	15	15	9	October 1938	8	0	0	0	do.
					December 1938	32	21	20	12	M (2)
Calcutta	26	15	15	10	February 1939	47	30	30	19	do.
TOTALS ..	51	33	33	22		98	58	57	37	..
PERCENTAGE	..	64·7	64·7	43·1		..	59·1	58·1	37·7	..

M (1) = Modification (1).   M (2) = Modification (2).

TABLE III.

Showing the comparative results of isolation of agglutinable vibrios in modified W & B's medium and alkaline peptone water from **contacts.**

Centre.	Date of contact with cholera case.	Contacts.				Date of examination.	Number of contacts examined.	Number of samples examined.	'O' SUB-GROUP I POSITIVE.			Type of W & B's medium used.
		Total number examined.	Total number of positive agg. vibrios.	In W & B's medium.	In 1 per cent peptone water.				Total number.	In W & B's medium.	In 1 per cent peptone water.	
Rampurhat	June 1934, April and September 1936.	12*	0	0	0	July 1938	12*	12	0	0	0	M (1)
Bashirhat Group A.	November 1931	36*	0	0	0	August 1938	36*	36	0	0	0	do.
Bashirhat Group B.	September 1938	21	4	4	4	September 1938	21	47	4	4	4	do.
						October 1938	17 (repetitions)	26	0	0	0	do.
TOTALS ..		69	4	4	4			121	4	4	4	
PERCENTAGE			5·8	5·8	5·8				3·3	3·3	3·3	

M (1) = Modification (1).

* *Cf.* postscript at the end of the paper.

TABLE IV.

Showing the comparative results of isolation of agglutinable vibrios in modified W & B's medium and alkaline peptone water from **non-contacts.**

Centre.	Date of examination.	NON-CONTACTS.				Number of samples examined.	'O' SUB-GROUP I POSITIVE.			Type of W & B's medium used.
		Total number examined.	Total number of positive agg. vibrios.	In W & B's medium.	In 1 per cent peptone water.		Number.	In W & B's medium.	In 1 per cent peptone water.	
Rampurhat	July 1938	54	0	0	..	54	0	0	..	M (1)
Rampurhat	December 1938	30	0	0	0	30	0	0	0	M (2)
Bashirhat Group A.	August 1938	85	0	0	..	85	0	0	..	M (1)
Bashirhat Group B.	September 1938	2	0	0	..	2	0	0	..	do.
TOTALS ..		171	0	0	0	171	0	0	0	

M (1) = Modification (1).   M (2) = Modification (2).

TABLE V.

Showing the comparative results of isolation of agglutinable vibrios in modified W & B's medium and alkaline peptone water from **water-supplies.**

Nature of source	Centre	Water-supplies: Total number examined	Water-supplies: Total number of positive agg. vibrios	Water-supplies: In W & B's medium	Water-supplies: In 1 per cent peptone water	Date of examination	Number of sources examined	Number of samples examined	'O' sub-group I positive: Total number	'O' sub-group I positive: In W & B's medium	'O' sub-group I positive: In 1 per cent peptone water	Types of W & B's medium used
Tanks	Rampurhat	26	1	1	1	July 1938	26	30	2	2	1	M (1)
	Rampurhat	11	0	0	0	December 1938	11	14	0	0	0	M (2)
	Bashirhat Group A.	33	1	1	1	August to September 1938.	33	46	2	2	1	M (1)
	Bashirhat Group B.	8	1	1	1	September to October 1938.	8	28	1	1	1	do.
	Calcutta	30	5	5	2	June to October 1938.	29	56	0	0	0	do.
	Calcutta					October 1938 to March 1939.	22 (repetitions)	68	5	5	2	M (2)
TOTALS		117	8	8	5		..	242	10	10	5	
PERCENTAGE		..	6·8	6·8	4·2		..	..	4·1	4·1	2·0	

	Locality				Date	(repetitions)					Remarks
Rivers	Rampurhat	1	0	..	July 1938	1	1	0	0	..	M (1)
	Bashirhat Group A.	1	0	..	August 1938	1	1	0	0	..	do.
	Calcutta	1	0	0	June to October 1938.	1	5	0	0	..	do.
					October 1938 to March 1939.	1 (repetitions)	8	0	0	0	M (2)
	TOTALS ..	3	0	0			15	0	0	0	0
Wells and Tube-wells.	Rampurhat	8	0	..	July 1938	8	8	0	0	..	M (1)
		1	0	0	December 1938	1	1	0	0	0	M (2)
	Bashirhat Group A.	2	0	..	August to September 1938.	2	2	0	0	0	M (1)
	TOTALS ..	11	0	0			11	0	0	0	0

M (1) = Modification (1). M (2) = Modification (2).

TABLE VI.

Showing the number and types of vibrios other than 'O' sub-group I isolated in the two media.

Sources. Nature.	Samples. Number.	Samples. Number examined in W & B's medium.	Corresponding number examined in 1 per cent peptone water.	NAG Isolations in W & B's medium.	NAG Isolations in 1 per cent peptone water.	Heiberg's Groups. Isolations in W & B's medium.						Heiberg's Groups. Isolations in 1 per cent peptone water.					
						I.	II.	III.	IV.	V.	VI.	I.	II.	III.	IV.	V.	VI.
Cases ..	51	98	98	13	35	9	3	1	0	0	0	22	9	1	0	3	0
Contacts ..	69*	121	71	18	15	9	2	4	0	3	0	5	4	4	0	2	0
Non-contacts	171	171	30	7	:	5	0	2	0	0	0	:	:	:	:	:	:
Tanks ..	117	242	108	135	106	111	18	0	0	6	0	79	24	0	0	3	0
Rivers ..	3	15	8	10	8	9	0	0	0	1	0	8	0	0	0	0	0
Wells ..	6	6	0	0	0	0	0	0	0	0	0	:	:	:	:	:	:
Tube-wells	5	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TOTALS ..	422	658	316	183	164	143	23	7	0	10	0	114	37	5	0	8	0

* *Cf.* postscript at the end of the paper.

TABLE VI-B.

Showing the comparative percentage and biochemical characters of the inagglutinable vibrios isolated in the two media.

Samples.	W & B's MODIFIED MEDIUM.							1 PER CENT PEPTONE WATER.						
	Number examined.	Number nag. +	Percentage.	MANNOSE + Number.	MANNOSE + Percentage.	MANNOSE − Number.	MANNOSE − Percentage.	Number of samples examined.	Number nag. +	Percentage.	MANNOSE + Number.	MANNOSE + Percentage.	MANNOSE − Number.	MANNOSE − Percentage.
Cases ..	98	13	13·2	10	77	3	23	98	35	35·7	26	74·2	9	25·8
Contacts ..	121	18	14·8	16	88·8	2	11·2	71	15	21·1	11	73·3	4	26·7
Non-contacts ..	171	7	4	7	100	0	0	30	..	..	..	..	..	..
Tanks ..	242	135	55·7	117	86·6	18	13·4	108	106	98·1	82	77·3	24	22·7
Rivers ..	15	10	66·6	10	100	0	0	8	8	100	8	100	0	0
Wells ..	6	0	0	0	0	0	0	..	..	..	..	..	..	..
Tube-wells ..	5	0	0	0	0	0	0	1	0	0	0	0	0	0
TOTALS ..	658	183	27·8	160	87·4	23	12·6	316	164	51·9	127	77·4	37	22·6

EXPERIMENTAL STUDIES IN PLAGUE.

Part I.

INTRODUCTION.

BY

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WHEN the present writer first became actively associated with the manufacture of antiplague vaccine twelve years ago the practice was in force of determining the protective value of the vaccine made at the Haffkine Institute, Bombay, by the method originally described by Morison *et al.* (1924*a*, *b*) and further worked out by Naidu *et al.* (1926). A batch of vaccine was not considered suitable for issue unless a certain percentage immunity was obtained by this test. The experimental animal employed was a susceptible strain of wild rat (*Rattus rattus*). As the Bombay city rat was found to have become very resistant to plague infection it was found necessary to import every month large batches of rats from Madras, a city where no serious epidemic of plague had occurred during the present pandemic. For testing the protective power of a vaccine a batch of 30 to 60 rats was used and 0·5 c.c. of the vaccine under test was administered subcutaneously to each animal. Seven days later each animal received subcutaneously a test infective dose, consisting of ' 0·003 milligram of the spleen of a rat that had died of acute plague '. The animals were then observed for a further period of 15 days and at the end of this period the number of animals surviving infection was noted and the protective power of the vaccine was expressed as ' percentage immunity ', i.e., the number of survivors as a percentage of the animals infected. As a certain number of rats died from the toxic effects of the large dose (0·5 c.c.) of the vaccine given, the animals which died during the interval between the administration of the vaccine and injection of the infective dose were excluded in calculating ' percentage immunity '. Ten rats were used as controls for each test. These were given the test infective dose but no vaccine. Between 80 and 90 per cent of these controls usually succumbed to the infection. By this method these workers had tested 14 batches of Haffkine's Plague Prophylactic and had obtained ' percentage immunity ' values

ranging from 13·9 to 52·7, with an average value of 36·8. On the basis of this work the method was adopted as a routine test for the standardization of the vaccine produced by the Haffkine Institute, and it was decided that no batch of vaccine was to be issued unless it had a 'percentage immunity' of not less than 25.

During the year 1928 eight representative brews of a huge production of 7,000,000 c.c. were tested by Naidu by his method and the following results were obtained (Table I) :—

TABLE I.

Results of routine testing of several brews of plague vaccine by the method of Naidu et al. (loc. cit.).

Number of brew.	'Percentage immunity.'
241	13·3
257	21·6
279	24·0
286	20·8
290	14·8
292	7·1
296	20·0
314	13·7

It would be seen that none of the brews tested during the year gave the required 'percentage immunity' of 25. Yet there was no reason whatsoever to believe that these brews were in any way different to those produced during the previous years. The methods of preparation had been standardized and were closely adhered to. The validity of the method of testing appeared to be open to doubt and no information was available as to its possible degree of error, the method having been based on single determinations on 14 brews. The demand at the time for the vaccine reached 7,000,000 c.c. in the year and if brews which did not come up to the standard of 25 per cent protection by the test employed and had been rejected it would not have been possible to meet it. Further, an alternative method of testing, hastily improvised, showed these brews to be possessed of good protective power.

The practice of issuing the vaccine only on the basis of the result of the test was accordingly abandoned and an investigation was instituted as to the validity of the method. In the first instance a series of duplicate tests were carried out on batches the results of which are given in Table II :—

TABLE II.

Results of duplicate determinations carried out to assess the error of the method of Naidu et al. (loc. cit.).

Number of vaccine.	Resultant 'percentage immunity'.	
	1st determination.	2nd determination.
60/4	17·8	19·23
65/4	13·6	44·00
61/3	13·6	21·42
64/3	56·6	17·39
60/3	44·4	10·71
62/4	65·2	34·90

These duplicate determinations showed a maximum variation of over 400 per cent in the testing of an individual vaccine. The second determinations were made six weeks after the first determination, yet the storage of the vaccines does not seem to have affected the results, because some vaccines gave a higher percentage immunity on second test. In fact, the results seemed to be merely chance results. On going through all the work reported by these authors it seemed that the test animal employed and the test infective dose (0·003 mg. of plague spleen per animal) were both at fault, and particularly so, the test infective dose. It was apparent that though the specifying of the test infective dose as '0·003 mg.' gave the impression of a great degree of accuracy it was really not so. The actual bacterial contents of seven test suspensions prepared by Naidu were counted and found to be 102, 5,435, 250, 15,600, 26,000, 40,000, and 32,000 organisms per c.c. (Sokhey and la Frenais, 1928). Besides this variation in the number of organisms contained in the test doses, there seems to have been involved some technical error in the preparation of the spleen emulsions. Naidu (1927) reported experiments carried out to 'determine the smallest amount of plague spleen which would produce a uniform mortality of 90 to 100 per cent in test (*Rattus rattus*) animals'. He found that 1 c.c. of the 1×10^{-22} dilution of the first suspension containing 3×10^{-3} mg. of plague spleen given subcutaneously per animal was enough to produce a mortality of 90 to 100 per cent. Later, Naidu and Jung (1929) in another experiment found that 1 c.c. of a 10^{-12} dilution of the original suspension containing

3×10^{-3} mg. of plague spleen was enough to kill 90 to 100 per cent of the rats inoculated. Otten (1933) repeated Naidu's experiments and found that even a 10^{-3} dilution of the original suspension produced a mortality of only 28·3 per cent and the next dilution (10^{-4}), a mortality of 1·4 per cent, while the original suspension produced a mortality of 98·7 per cent.

On the evidence of the duplicate determinations given above and other relevant matters a committee of plague workers at the Haffkine Institute which met in 1929 decided that the method was seriously at fault and should not be employed for the standardization of the vaccine. This decision was inevitable but left matters in a very unsatisfactory state. The only sanction we had for the then methods of preparation of Haffkine vaccine was based on the results of the experimental work carried out between 1924 and 1929 by employing this method (Taylor, 1933). The methods of preparation and in fact the protective power itself of the vaccine had been in dispute for some years and in 1924 the Indian Research Fund Association financed an inquiry to investigate the whole matter. The experimental work thus carried on the basis of the method developed by Naidu *et al.* (*loc. cit.*) had supported the existing practice as regards the selection of the seed, the duration of incubation of broth cultures, pH of the broth, and the very form of the vaccine. When it was found that the method of measuring the protective power of the vaccine was very faulty, all the superstructure built on it went down and left the position as it was in 1924. A fresh investigation was imperative as no practice connected with the preparation of the vaccine seemed to be founded on any very secure scientific grounds, as perusal of the literature abundantly showed.

CONFLICTING VIEWS ON HAFFKINE VACCINE.

Haffkine (1897) originally, on purely theoretical grounds, decided to grow the plague organism in nutrient broth instead of growing it on solid medium for the preparation of his plague vaccine. He assumed ' there is the possibility of the bactericidal power being created by the injection of the bodies of the microbes, or substances enclosed in them, while antitoxic properties may be communicated by the injection of the metabolic substances secreted or produced in the surrounding media '. He had carried out no experiments to support this assumption. In fact he could not have done so, for he started work on the preparation of his prophylactic on the 8th October, 1896, and was ready with it and had started using it on 16th January, 1897. However, Bannerman (1902) stated, ' Haffkine was successful in protecting rabbits against an inoculation of virulent plague microbes, by treating them previously with a subcutaneous injection of a culture in broth of the organisms sterilized by heat '.

Haffkine's position was soon assailed by Gaffkey *et al.* (1899) working with the monkey (*Macacus radiatus*) and also by Fraser *et al.* (1900) working with the guinea-pig, who showed that the clear limpid fluid of Haffkine vaccine conferred absolutely no protection against plague. Kolle and Otto (1903, 1904) went much further and asserted, on experimental basis, that a heat-killed vaccine had no protective power whatsoever. The value of Haffkine vaccine was, however, claimed to be based on the results of the use of the vaccine in the field. The statistical data of the results of inoculations collected in India soon after the introduction of the use of the vaccine

(Taylor, *loc. cit.*) did undoubtedly tend to show, in spite of their statistical short-comings, that Haffkine plague vaccine was possessed of good protective value. But the position was unsatisfactory, because several changes in the methods of preparation of the vaccine had been introduced from time to time as will be indicated below and no satisfactory measurement could be made of the effect of these changes on the resultant vaccine. The position, confused as it was, was made worse by the publication of the results of an extensive field trial of Haffkine vaccine carried out in Java in 1921–1922, which suggested the vaccine to be possessed of poor protective power. Otten (1936) summarized these results and showed that it (Haffkine vaccine) reduced plague mortality only to half, while the previous field tests in India had shown six to tenfold reduction of mortality. It is to be noted, however, that only a part of the vaccine used in Java was supplied by the Haffkine Institute, the rest was made in Java itself but no details are available of its exact mode of preparation.

The position of Haffkine vaccine was further attacked by Kolle and Otto (*loc. cit.*) who showed that as contrasted with heat-killed vaccines which had little or no protective value live vaccines prepared from attenuated strains were possessed of very high protective power. Strong (1907) confirmed these findings and carried these experiments further and successfully used these living vaccines for inoculating human beings with good results. In more recent times Girard and Robic (1934) and Otten (1936) have tested living vaccines by extensive inoculations with very good results. Otten on the basis of his work has asserted 'no dead vaccine whatever conferred satisfactory protection on guinea-pigs and wild rats— the test animals most susceptible to plague infection' and has advocated the use of living vaccines.

SOME CHANGES INTRODUCED IN THE PREPARATION OF HAFFKINE VACCINE FROM TIME TO TIME.

The value of Haffkine vaccine was based on the statistical evidence of inocula-tions carried out in the field soon after the vaccine was introduced. But since that period several changes had been introduced in the methods of its preparation. Haffkine started making his vaccine from strains of *Past. pestis* freshly isolated from fatal human cases. But he soon found that it was not always possible to get fresh strains and resorted to passage through guinea-pigs. After he left the Haffkine Institute the practice of passaging strains through susceptible wild house rats was started and was continued till 1933. There is no clear evidence as to what happens to the virulence and antigenic value of strains when passed through guinea-pigs, but in the case of rats, Burgess (1927) has shown that under certain conditions passage through a rat lowers the virulence of a strain.

As regards the period of incubation of broth cultures the changes introduced were even greater. Haffkine incubated his broth cultures for six weeks at room temperature (i.e., 26°C. to 29°C.). The later directors of the Institute varied the incubation period between two weeks and nine months (Taylor, *loc. cit.*). These wide variations in the period of incubation must have produced some differences in the protective power of the resultant vaccines but no experimental evidence is available assessing the value of these changes.

Another important aspect in which the preparation of Haffkine vaccine has been varied from time to time has been the temperature at which the broth cultures were killed and the duration of time for which the heat was applied. Haffkine (1897) first killed his cultures at 70°C. and applied this heat for one hour. Two years later he started killing his cultures between 50°C. and 55°C. for 15 minutes (Haffkine, 1899) and the records of the Haffkine Institute show that after Haffkine left the Institute in 1905, the killing temperature was raised to 60°C. to 64°C. and was applied for 15 minutes. This practice was followed till the end of 1931. Obviously these temperatures must have profoundly affected the resultant vaccines, but for lack of suitable methods of measuring the effects of these changes, the matter remained uninvestigated.

The position of the preparation of Haffkine vaccine at the end of 1928.

Thus, in 1929 the technique developed by Haffkine for the preparation of the vaccine was followed on its general lines, but there was doubt as to its exact immunizing value and the precise effect of the various factors involved in its preparation. It is true that a great deal of experimental work had been published on the subject in India and abroad, but the conclusions arrived at were very conflicting. Different workers had employed such a multiplicity of entirely unstandardized methods of testing that the experiments of one worker could not be compared with the work of another worker. It seems the main difficulty of previous workers was due to the lack of a constant test infective dose, constant both as regards virulence and number of organisms. Petrie (1929) writing about the same time as the present work was commenced stated, ' There is no standard method of testing for virulence ' of the plague organism.

There were thus many points in regard to the vaccine which had not been determined or only tested on the basis of an unreliable method. These included :—

(a) The existence of *pestis* strains of different antigenic types and, if such existed, their value for manufacture of vaccine.

(b) The advisability of using strains isolated from human cases or maintenance of strains by animal passage.

(c) The suitability of virulent or avirulent strains.

(d) The advantages or disadvantages of liquid or solid media for growth.

(e) The most suitable temperature and duration of incubation.

(f) The method of killing the organism including the effect of heat and preservatives on protective value.

The additional question has further been raised in recent years as to the relative value of heat-killed vaccine made from virulent strains as compared with the use of live avirulent suspensions. Before these questions could be answered it was obviously necessary to develop a reliable method of testing the protective power of the vaccine and work on this subject was commenced in 1928. Progressive accounts of this work have already been published in the Haffkine Institute Reports commencing with the year 1928, and short summaries have also been reported elsewhere (Sokhey, 1932, 1936 ; Sokhey and Maurice, 1935, 1937 ; Sokhey and Chitre,

1937). It is now proposed to present in this series of communications an account of the methods developed and the results obtained over a considerable period of years on the related subjects of plague vaccine, antiplague serum, chemotherapy of plague, and the changes in the susceptibility of rats in areas where plague epidemics have raged.

SUMMARY.

In the absence of a reliable method of estimating the protective value of the antiplague vaccine experimentally it has not been possible to determine the effect of different factors entering into its manufacture. The position is outlined prior to an account of further investigations.

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EXPERIMENTAL STUDIES IN PLAGUE.

Part II.

THE SOLID MEDIUM OF CHOICE, AND THE OPTIMAL TEMPERATURE OF INCUBATION, FOR THE GROWTH OF THE PLAGUE BACILLUS.

BY

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ONE of the essentials for protection experiments in animals is the availability of a constant infective test dose—constant both as regards virulence and the quantity of organisms. The most appropriate way to particularize a quantity of organisms is to give their number. Workers have been content to use rough and ready methods of specifying the quantity of organisms used for inducing infection in their experimental animals. Early workers, Gaffkey *et al.* (1899), Albrecht and Ghon (1900), Kolle and Otto (1903), and Kolle *et al.* (1904) expressed the quantities of organisms used by them for an infective dose as a loopful or portion of a loopful of a growth from a nutrient-agar slope. The British Plague Commission (1912) used a given weight of the spleen of a rat dying of acute plague. Rowland (1910) used 1/10 c.c. of a broth growth, and Schutze (1925) described his infective dose as $\frac{1}{2}$ c.c. of a two-day broth culture, and later this worker used a weighed amount of plague spleen and specified the number of organisms contained in it, but the author does not indicate the method of enumeration employed by him (Schutze, 1932). In the Haffkine Institute the standard infective dose consisted of 0·003 mg. of plague spleen of a rat dying of acute plague.

Indeed the literature contains few records of actual counts of plague organisms. The British Plague Commission (1906) made very rough enumerations of a number of organisms in the blood of a rat dying of acute plague by the dilution method in nutrient broth. Liston (1906) reported a count made on a 48-hour culture of plague in broth by plating successive broth dilutions on nutrient-agar slopes. He planted 10 c.mm. of each successive tenth dilutions on an agar slope, and found that he obtained 2 to 4 colonies in the 1 in 100,000 dilution. Taking an average of 3 colonies in 10 c.mm. of a 1 in 100,000 dilution would give a count of 30 millions per c.c. in the original 48-hour broth growth. As will be shown later this count was roughly one-tenth to one-twentieth of what it should be. Besides this inaccuracy, he must

have found the successive counts very variable as did Stevenson and Kapadia (1924) (work done in 1910–11, but reported in 1924) who, working in Liston's laboratory, stated ' attempts were made to count the number of bacilli so injected by the dilution process and subsequent growths on agar slopes, but owing to the auto-agglutination of plague bacilli in suspension such irregular counts were met with that the attempts had to be abandoned '. Later, D'Aunoy (1923) reported a large number of counts from 80-hour growths in broth incubated at 28°C. to 30°C. He plated his dilutions on nutrient-agar plates for colony counts. He does not say whether he encountered any difficulty with regard to the constancy of results such as was met by Stevenson and Kapadia.

THE MEDIUM OF CHOICE FOR THE GROWTH OF *Pasteurella pestis.*

The difficulties encountered in the enumeration of viable plague organism by the method of plating were due to the use of an unsuitable medium. All the workers had assumed, and it now appears quite wrongly, that nutrient agar was a suitable medium for the growth of the plague organism. Textbooks on bacteriology have taught that *Past. pestis* grows well on ordinary nutrient agar. In the course of the present studies undertaken by the writer on the subject of a test infective dose, attempts were first made to estimate the number of organisms in 48-hour broth cultures by making successive 1/10 dilutions and plating on ordinary nutrient agar. With a dilution of 10^{-1} a confluent growth would result and with 10^{-2} the growth would be very crowded, while with 10^{-3} no growth at all would appear after 48 hours' incubation at 27°C., the optimal temperature of incubation for the organism in liquid media. Gore (1929*a*, *b*) working on the problem at about the same time at the Haffkine Institute observed that the addition of blood to nutrient agar promoted the growth of *Past. pestis.* High dilutions of broth growths or suspensions of *Past. pestis*, which would show no growth when planted on nutrient-agar slopes, would produce a number of discrete colonies when planted on blood-agar slopes. He took advantage of this differential growth of the organism on the two media for devising a test for testing the purity of plague cultures. The test he devised consisted in the planting of a high dilution of broth culture or suspension under test on nutrient-agar slopes and blood-agar slopes, and incubating them at 37°C. for 48 hours. At the end of this period of incubation the agar slopes should show no growth at all if the original cultures are pure, while blood-agar slopes should show discrete colonies of *Past. pestis.* The development of any colonies at all on agar slopes makes suspect the purity of the original culture. The test in detail is described by Taylor (1933), and has, with great saving of labour, been in routine use at the Haffkine Institute since the middle of 1928.

On the basis of this observation blood agar was used for colony counts and the successive dilutions were found to give closely comparable results. Later, Schutze and Hassanein (1929) reported observations attributing the difficulty of initiating growth of plague and other *Pasteurella* strains lying widely scattered on agar plates to oxygen sensitivity of these organisms. In their opinion the addition to agar of reducing substances overcame this difficulty. These observations were further extended by Wright (1934).

The relative growth of the plague bacillus on nutrient agar, 5 per cent and 0·1 per cent blood agar, agar containing 0·05 per cent copper sulphate, and agar with 0·05 per cent sodium sulphite, was estimated. For preparing blood agar, nutrient agar was melted and allowed to cool to 45°C. and then the desired amount of defibrinated rabbit blood was added, thoroughly stirred, and poured into Legroux tubes, 220 mm. long and 25 mm. in diameter, each tube giving a usable spreading surface of about 40 sq. cm. These tubes are illustrated in Plate XII. Working in a highly industrialized Indian city these tubes were found to be a great improvement on Petri dishes, as they made it easy to eliminate aerial contaminations.

For measuring the relative growth of *Past. pestis* on the various media mentioned above, each of the seven progressive tenth dilutions in nutrient broth of a 48-hour broth culture of a fully virulent strain incubated at 27°C. was plated on a set of eight parallel tubes of each medium with a special pipette designed to deliver the dilution and also to spread it on the surface of the medium. This pipette will be described in Part III of this communication (page 332). 0·05 c.c. of a given dilution was seeded in each tube. Legroux tubes after being seeded were incubated for 72 hours at 37·5°C. The results obtained are given in the Table :—

TABLE.

Growth of Past. pestis *on agar surfaces.*

Seven progressive tenth dilutions were made from a 48-hour broth culture of a fully virulent strain incubated at 27°C. Sets of eight parallel plates of each medium were seeded with each dilution; 0·05 c.c. of dilution being spread on each plate. Legroux tubes were employed and the amount of dilution used was spread on about 40 sq. cm. of the agar surface. The tubes after being seeded were incubated for 72 hours at 37·5°C.

| Medium.	Average number of colonies per tube in the various dilutions used.						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Nutrient agar	Confluent growth.	104	3	1	0	0	0
5 per cent rabbit-blood agar	,,	Profuse growth.	Profuse growth.	Profuse growth.	142	18	1
0·1 per cent rabbit-blood agar	,,	,,	,,	,,	135	18	1
10 per cent horse-serum agar	,,	,,	,,	,,	130	16	1
0·05 per cent copper-sulphate agar.	,,	,,	,,	,,	112	12	1
0·05 per cent sodium-sulphite agar.*	,,	,,	,,	,,	82	7	0

* The sulphite-agar tubes were prepared 36 hours before use. The reaction of all media was adjusted at pH 7·0.

These experiments have been repeated a number of times with similar results and leave no doubt about the fact that sparsely seeded *Past. pestis* does not grow on nutrient-agar surface. If blood serum, copper sulphate, or sodium sulphite is added to nutrient agar, growth of the organism is enhanced, but the enhancement of the growth in the case of copper sulphate, sodium sulphite, and serum is not always as consistent as is shown in the Table. Now and then the enhancement is much less than in the case of blood. From extensive experience of the subject it has been found that blood agar is the best solid medium for the growth of *Past. pestis*. When high dilutions of a suspension of broth culture are planted on this medium the growth is maximal and the discrete colonies resulting equal approximately to the number of organism present in the dilution. This point will be dealt with more fully in a later communication.

OPTIMAL TEMPERATURE OF INCUBATION FOR THE GROWTH OF PLAGUE BACILLUS ON SOLID MEDIA.

Precise measurements of the growth of the plague organism in nutrient broth at different temperatures from 0°C. to 45°C. have been made (Sokhey, 1938) and these findings will be given in a separate communication; for the present it will suffice to state that for a 48-hour period of incubation maximum growth in broth takes place between 23°C. and 27°C. However, when high dilutions of plague-broth cultures are seeded on agar slopes, no growth results if the slopes are incubated at 27°C., while some growth appears if they are incubated at 37·5°C. If progressive tenth dilutions of a broth culture of a fully virulent strain grown at 27°C. for 48 hours are made in nutrient broth and 0·05 c.c. of each dilution is planted on about 40 sq. cm. of an agar surface and incubated for 48 to 72 hours at 27°C., a profuse confluent growth results from dilutions 10^{-1} and 10^{-2}, but no growth at all appears on surfaces seeded with dilutions higher than 10^{-2}. That is at 27°C. temperature of incubation either profuse confluent growth results or no growth at all. No discrete colonies can be obtained. A plague-broth culture grown for 48 hours at 27°C. contains approximately 400 millions organisms per cubic centimetre. 0·05 c.c. of dilution 10^{-2}, which gives a confluent growth, would therefore contain 200,000 organisms, and since 0·05 c.c. of the dilution is spread on 40 sq. cm., it would appear that about 5,000 organisms must be planted on each sq. cm. of an agar surface to get any growth at all, if the plates are incubated at 27°C. The phenomenon is illustrated in Plate XII. Legroux tubes from left to right were seeded with 0·05 c.c. of dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} of a 48-hour broth grown at 27°C. and were incubated at 27°C. for 96 hours. In the first tube, with 10^{-2} dilution, a thick growth resulted, but in the other tubes no growth at all appeared. Even incubation for a further period of 72 hours did not result in any growth in the last three tubes.

When however Legroux tubes were seeded with same dilutions and in the same quantities and at the same time as those reproduced in Plate XII, but incubated at 37·5°C. for 96 hours, discrete colonies appeared even in the tube seeded with dilution 10^{-3}. This is not a chance result. In the course of the work very many

PLATE XII.

Dilution 10^{-2}

Dilution 10^{-3}

Dilution 10^{-4}

Dilution 10^{-5}

Temp 27°C Nutrient Agar

Temp 27°C Nutrient Agar

Temp 27°C Nutrient Agar

Temp 27°C Nutrient Agar

PLATE XIII.

Dilution 10^{-2}

Dilution 10^{-3}

Dilution 10^{-4}

Dilution 10^{-5}

Temp 37·5°C
Nutrient Agar

Temp 37·5°C
Nutrient Agar

Temp 37·5°C
Nutrient Agar

Temp 37·5°C
Nutrient Agar
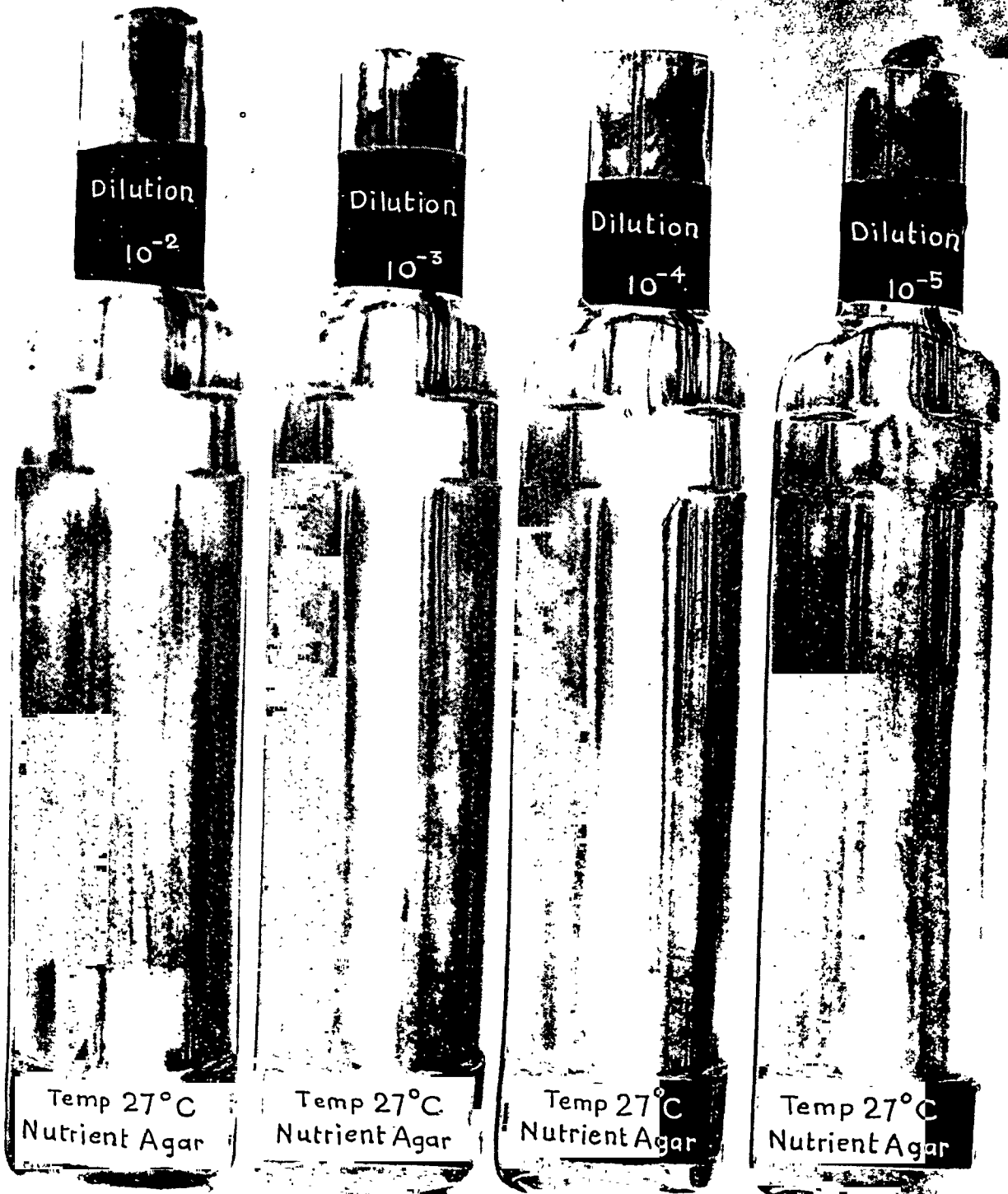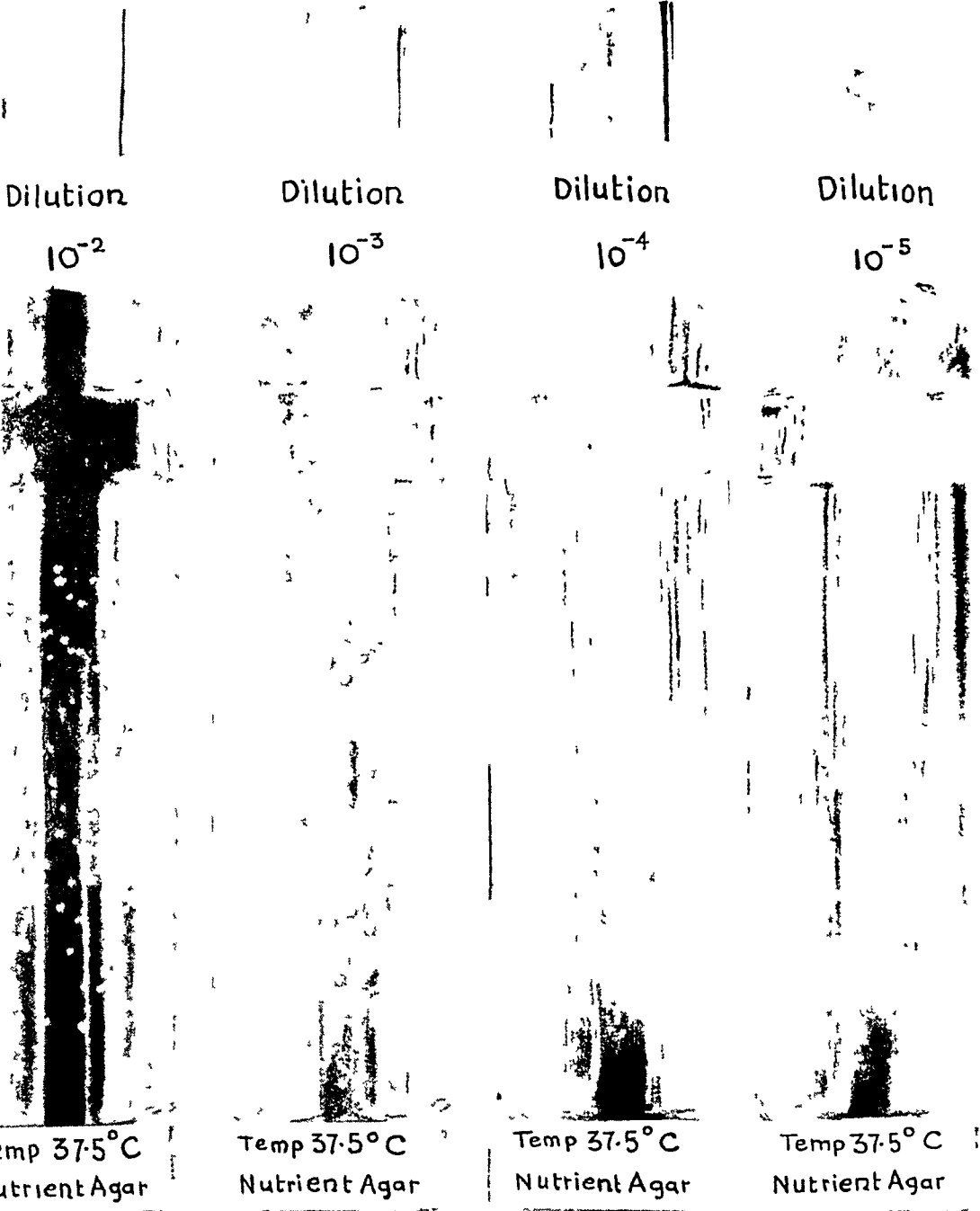
EXPLANATION OF PLATES XII AND XIII.

The effect of temperature of incubation on the growth of plague bacillus on nutrient-agar surfaces.

Two sets, each of four Legroux tubes, of nutrient agar were planted at the same time with dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} of a 48-hour broth culture grown at 27°C. 0·05 c.c. of each dilution was planted per tube. One set was incubated at 27°C. and the other at 37·5°C. for 96 hours.

Plate XII shows the set incubated at 27°C. for 96 hours. There is profuse growth in the first tube seeded with 10^{-2} dilution and no growth at all in other tubes.

Plate XIII shows the set incubated at 37·5°C. for 96 hours. Discrete colonies appear in the first and second tubes (144 and 32 in number) seeded with dilution 10^{-2} and 10^{-3} respectively, and no growth in third and fourth tubes. Note that although at this temperature of incubation growth appears in the tube with dilution 10^{-3}, while there was no growth in the corresponding tube when incubated at 27°C., yet the growth in the first tube with dilution 10^{-2} is less profuse than in the corresponding tube when incubated at 27°C.

Growth of the plague bacillus on blood-agar plates incubated at 37·5°C.

To compare the growth of *Past. pestis* on blood agar with that on nutrient agar, a set of seven Legroux tubes was seeded with dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} of a 48-hour broth culture grown at 27°C., by spreading 0·05 c.c. of each dilution on about 40 sq. cm. of 5 per cent rabbit-blood agar surface per tube. These tubes were incubated for 96 hours at 37·5°C., and can be compared with those in Plate XIII. In the case of blood agar, growth appears in dilutions up to 10^{-7}. Plate XIV shows blood-agar tubes with dilutions 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, while Plate XV shows tubes with dilution 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. It is to be noted that the fourth tube in Plate XIV and the first tube in Plate XV is the same tube. The growth is too profuse to count in the first three tubes in Plate XIV, representing dilutions 10^{-2}, 10^{-3}, and 10^{-4}. The growths in tubes with dilution 10^{-5}, 10^{-6}, and 10^{-7} in Plate XV are discrete colonies numbering 161, 21, and 2 respectively. There is no growth in tube with dilution 10^{-8}.

PLATE XIV.

Dilution 10^{-2}

Dilution 10^{-3}

Dilution 10^{-4}

Dilution 10^{-5}

Temp 37·5°C
Blood Agar

Temp 37·5°C
Blood Agar

Temp 37·5°C
Blood Agar

Temp 37·5°C
Blood Agar

PLATE XV.
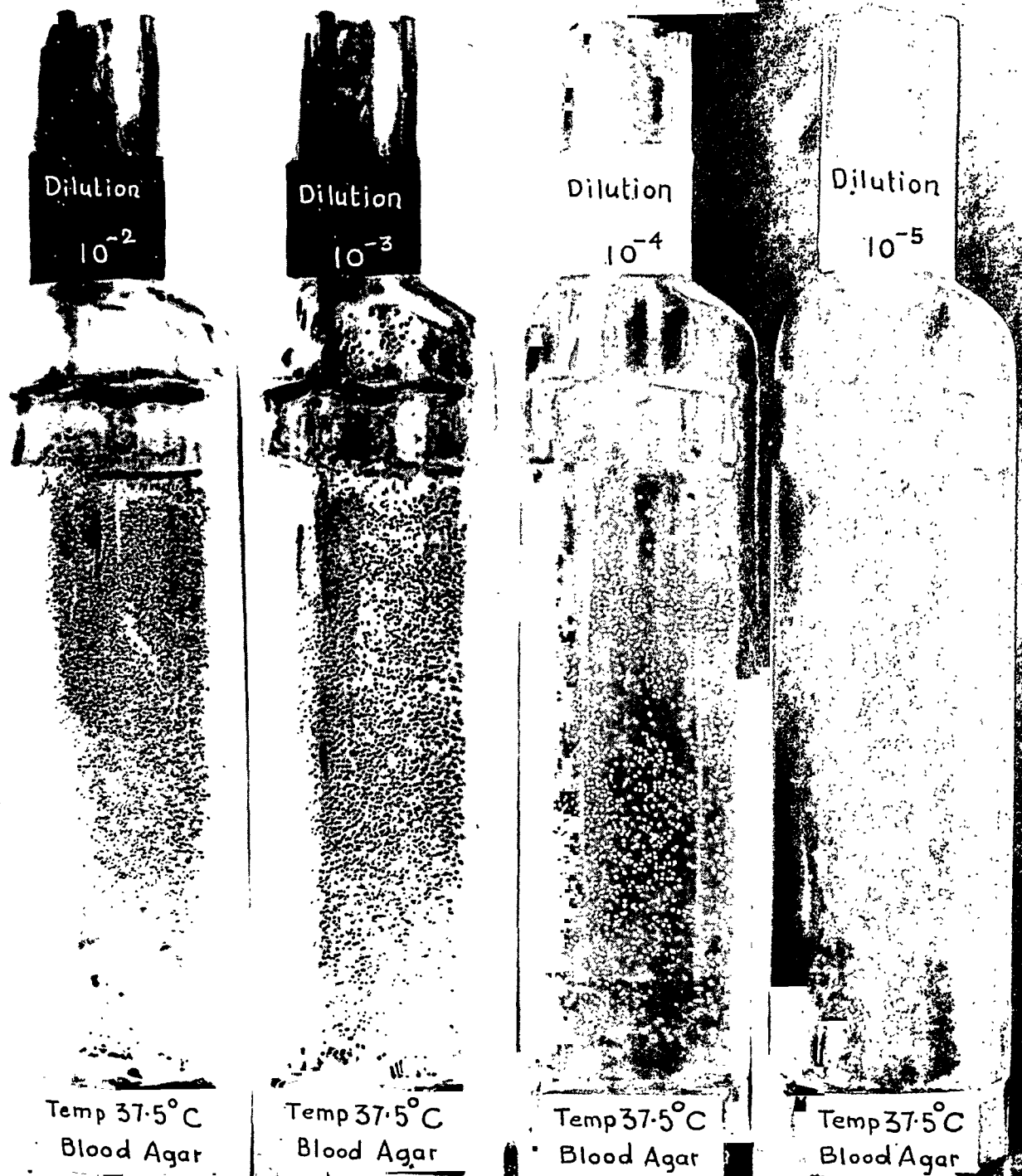
Dilution	Dilution	Dilution	Dilution
10^{-5}	10^{-6}	10^{-7}	10^{-8}

Temp 37·5°C
Blood Agar

Temp 37·5°C
Blood Agar

Temp 37·5°C
Blood Agar

Temp 37·5°C
Blood Agar

determinations have been made and very often growth appears even in 10^{-4} and 10^{-5} dilutions when incubated at 37·5°C. For example, in the experiment given in Table I growth on nutrient agar appeared in dilution 10^{-4}. It is, however, to be noted that in the tube seeded with dilution 10^{-2}, the growth is in discrete colonies and definitely less than the growth in the corresponding tube which was incubated at 27°C. The phenomenon is illustrated in Plate XIII. That growth in the tube seeded with 10^{-2} dilution should be more when incubated at 27°C. than when incubated at 37·5°C. is in keeping with the observation that the optimal temperature of growth for the plague organisms in nutrient broth is in the neighbourhood of 27°C. Yet no growth appears in the tubes with 10^{-3} dilution when incubated at this temperature, while appreciable number of discrete colonies appear when incubated at 37·5°C., a temperature at which growth in a liquid medium is less than 1/10th of the growth at 27°C. The fact is undoubted that at the incubation temperature of 27°C., the optimal temperature of growth of the organism in a liquid medium, about 5,000 organisms per sq. cm. of nutrient-agar surface must be planted to yield any growth at all. While at the incubation temperature of 37·5°C., a temperature very unfavourable for the growth of the organism in a liquid medium, 500 organisms planted per sq. cm. of nutrient-agar surface are enough to yield some growth though the growth is only about one-thousandth of the possible growth.

At the same time as Legroux tubes of nutrient agar, photographed in Plates XII and XIII, were seeded two sets each consisting of seven blood-agar Legroux tubes were also seeded to contrast the growth on this medium with the growth on nutrient agar. One set was incubated at 27°C. and the other at 37·5°C. The set of tubes incubated at 37·5°C. is photographed in Plates XIV and XV. It will be seen that in the case of blood agar as high a dilution as 10^{-7} of the same 48-hour broth culture yielded some colonies. The set of tubes incubated at 27°C. gave almost the same number of colonies in the respective dilutions; but the colonies were slightly smaller. The growth on blood agar, whether incubated at 27°C. or 37·5°C., is maximal, and the number of colonies resulting approach very closely to the number of organism present in a dilution.

DISCUSSION.

Experiments are described elucidating the effects of two temperatures of incubation, 27°C. and 37·5°C., on the growth of *Past. pestis* on nutrient agar-surfaces. It is shown that if progressive tenth dilutions are made of a 48-hour broth culture grown at 27°C., containing about 400 millions organisms per c.c., and 0·05 c.c. of each dilution is planted on 40 sq. cm. of nutrient-agar surface and the plates are incubated at 27°C. confluent growth appears in the tube seeded with 10^{-1} and 10^{-2} dilutions, but no growth appears on tubes seeded with dilutions 10^{-3} and higher. If the tubes are incubated at 37·5°C., some growth in the form of discrete colonies results even in the tubes seeded with 10^{-3} though the growth in the tube seeded with 10^{-2} dilution is less than the growth in the corresponding tubes incubated at 27°C. That in the latter case, i.e., incubation at 27°C. of tube with 10^{-2} dilution,

there should be more growth is understandable because maximum growth in broth takes place in the neighbourhood of this temperature, but question arises why the tube seeded with 10^{-3} dilution should show growth when incubated at 37·5°C. and not when incubated at 27°C. because in the case of the growth of *Past. pestis* in broth only about 1/10th as much growth results at this temperature as at 27°C.

Whatever the explanation, the fact remains that when plates are incubated at 27°C. about 5,000 organisms per sq. cm. of the nutrient-agar surface must be planted to get any growth at all, but when the plates are incubated at 37·5°C. seeding of 500 organisms per sq. cm. of nutrient-agar surface is enough to give growth. It is, however, to be noted that in sparsely seeded tubes though the growth at 37·5°C. is more than at 27°C. it is still only about one-thousandth of the possible growth.

The addition to nutrient agar of rabbit blood (5 per cent to 0·1 per cent), horse serum (10 per cent), copper sulphate (0·05 per cent), or sodium sulphite (0·05 per cent) makes the growth maximal. Of the various agents mentioned, blood when added to agar consistently gives reliable results and the numbers of colonies resulting on this medium from the seeding of dilutions tend to equal the actual numbers of organisms present in the dilutions.

The increase in the growth of *Past. pestis* resulting from the addition of blood, copper sulphate and sodium sulphite has been attributed to the reducing power of these agents. This explanation hypothesizes that *Past. pestis* is oxygen sensitive and the addition of reducing substances lowers the oxygen tension obtaining in an ordinary agar plate and thereby permits *Past. pestis* to grow. The better growth noted in sparsely seeded tubes when incubated at 37·5°C. than when incubated at 27°C. probably suggests that some other factor or factors are also involved, unless it so happens that 10° rise of temperature lowers the oxygen tension of the medium sufficiently to permit of growth, which is not very likely. There is another common observation which militates against this hypothesis, i.e., when *Past. pestis* is grown in a liquid medium like nutrient broth the total growth takes place at the surface of the medium where the medium is in contact with oxygen and never in the body of the medium. Similarly, the better growth on serum agar does not lend support to the oxygen tension theory.

SUMMARY.

Experiments are reported which confirm the observation that the addition of defibrinated blood, serum, copper sulphate, and sodium sulphite promotes the growth of sparsely seeded *Past. pestis* on agar surfaces. It is indicated that the possible reduction of oxygen tension on agar surfaces by the addition of reducing agents is not the sole explanation of the increased growth. Though an explanation of the observed facts is uncertain, it is clear that for the cultivation, on solid medium, of sparsely seeded *Past. pestis*, the medium of choice is blood agar, and the optimal temperature of incubation 37·5°C., a temperature very unfavourable for the growth of this organism in a liquid medium.

ACKNOWLEDGMENT.

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EXPERIMENTAL STUDIES IN PLAGUE.

Part III.

A METHOD FOR DETERMINING THE NUMBER OF VIABLE PLAGUE ORGANISMS IN BROTH CULTURES.

BY

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In the previous part of this paper (Sokhey, 1939) the view was stressed that one of the essentials of animal experimental work is the availability of a constant infective dose, constant both as regards virulence and the quantity of organisms, and that the most appropriate way to particularize a quantity of organisms is to give their number. The defects of previous attempts at enumerating the number of organisms present in a suspension or in a broth culture by colony-count method were shown to be due to the use of nutrient agar for plating, a medium unsuitable for the growth of *Past. pestis.* It was also shown that blood agar was the medium of choice and a temperature of 37·5°C. the optimal temperature for the growth of the plague bacillus sparsely seeded on solid media. On the basis of these observations a method for counting viable organisms contained in a broth culture or in a suspension by plating has been worked out, the details of which will be described in this paper.

The method was utilized for the purpose of developing a constant infective dose from 48-hour broth cultures incubated at 25°C. to 27°C. and these will be chiefly referred to, but the method is equally applicable to suspensions of the organism made from growths on solid media.

Ten c.c. of the broth culture, the bacterial content of which is desired to be measured, are introduced into a 20-c.c. ampoule with a flat bottom and the ampoule sealed. Then the ampoule is vigorously shaken to break up clumps and chains of the organism. For this purpose the ampoule is shaken in the shaking attachment of an International Centrifuge (International Equipment Co., Boston, U.S.A.) for five minutes. The broth in the course of shaking develops a considerable amount of froth. To make the froth subside it is touched with a straight platinum wire dipped in octyl alcohol for about 1 cm. of its length. The

addition of this amount of octyl alcohol does not interfere with the viability of the organisms. Next a series of progressive tenth dilutions are made by adding 1 c.c. of the culture to nutrient broth with standardized 1-c.c. pipettes graduated to deliver. For each dilution a fresh 1-c.c. pipette is used and the fluid from the pipette is delivered sufficiently slowly to prevent droplets of fluid forming and remaining behind in the lumen of the pipette. Immediately before a pipette is charged, the suspension is thoroughly shaken for about three minutes by rotating the tube between the palms of the hands.

Of the dilutions so prepared, a dilution or dilutions are selected for plating on blood agar such as will give discrete colonies when 0·05 c.c. of the dilution is spread on about 40 sq. cm. of the blood-agar surface and incubated at 37·5°C. Each of the selected dilutions is planted on a set of eight blood-agar (5 per cent rabbit blood) Legroux tubes (each 220 mm. × 25 mm.). Each tube is planted with 0·05 c.c. of a dilution. This quantity of a dilution is delivered and spread on about 40 sq. cm. of the surface of the blood agar in the Legroux tube with a special pipette.

This special pipette is 25 cm. in length and 0·8 cm. in external diameter. It has a largish bore, and the lower 15 cm. of the pipette is graduated to the tip into 0·05 c.c. divisions to deliver 0·5 c.c. in all. The last 2 cm. of the tip end of the pipette is ground into a cone to permit the pipette being used as a spreader. Thus, the pipette is used both to deliver the inoculum and to spread it on the surface of the medium.

0·05 c.c. of a dilution is delivered on to the middle of the blood-agar surface of the Legroux tube and is carefully spread by means of the cone end of the pipette on about 40 sq. cm. of the surface. For spreading the inoculum the cone end of the pipette is systematically passed over the area at least eight times. The quantity of the inoculum per tube (40 sq. cm. blood-agar surface) was fixed at 0·05 c.c. because it was found that this quantity was just enough to cover the area without permitting any of the inoculum to run on to the edges of the surface.

The selection of a suitable dilution for plating is a matter of importance. It is found that a dilution of which 0·05 c.c. when spread over 40 sq. cm. of blood-agar surface produces between 10 and 40 colonies gives the maximal count. If the next lower dilution is used the counts tend to be low, and if the next higher dilution is used the counts tend to be somewhat irregular. For a 48-hour broth culture incubated at 27°C., the 10^{-6} dilution answers the requirements best. In practice it is enough to plate this dilution alone for colony counts; the dilution above and below this, however, may be plated to check the count of the 10^{-6} dilution.

THE ACCURACY OF COUNTS.

The accuracy of the colony counts made by this method can be assessed from two different points of view. For certain experimental purposes, it is important to know that if the same batch of broth and the same strain of organism are used and 48-hour cultures are made under exactly the same conditions on two or more different occasions, how close the quantitative measurements of the resultant growths by this method are? In·this connection the requirement 'under exactly the same conditions' must be clearly defined. Ordinarily, if the quantity of the

nutrient broth of the same batch, the amount of inoculum and the temperature of incubation are kept constant the resultant growths can be expected to be approximately equal, but this is not so in the case of *Past. pestis.* The difference is created by the fact *that* Past. pestis *grows only on the surface of the liquid medium* and not in the body of the medium. Therefore, the total quantity of the broth used is not as important as the surface area of the broth, or rather the circumference of the surface area, as will be shown below. Thus, if each of the two test-tubes of a certain diameter is filled with 10 c.c. of broth and one is kept absolutely vertical and the other at an angle, the growth in the slanting tube is much more than the growth in the vertical tube. For small surface areas the growth appears to be directly proportional to the surface area, as is shown by the following experiment :—

Two test-tubes of exactly the same size (150 mm. long and 18 mm. internal diameter) were taken and 10 c.c. of broth of the same batch placed in both of them. These tubes were seeded with 0·05 c.c. of a 48-hour broth culture and rotated to thoroughly mix the inoculum with the broth. The two tubes were then placed in an incubator adjusted to 25°C., one tube was kept absolutely vertical and the other was kept slanting, and the edges of the slant were marked on the sides of the test-tube. The surface area of the broth in the vertical tube was 2·98 sq. cm. and in the slanting tube 6·45 sq. cm. The tubes were incubated for exactly 48 hours and the number of organisms per c.c. of broth in the two tubes was counted by the method described above. The results obtained are given in Table I :—

TABLE I.

Effect of surface area and its circumference on the growth of
Past. pestis *in nutrient broth.*

Ten c.c. of nutrient broth were placed in each of two test-tubes of exactly the same internal diameter. One tube was incubated in a vertical position and the other in a slanting position for 48 hours at 25°C.

Position of the test-tubes in the incubator.	Size of the test-tubes, mm.	Quantity of broth, c.c.	Surface area of broth, sq. cm.	Circumference of surface area of broth, cm.	Total growth in millions of organisms.	Growth per c.c. of broth in millions of organisms.	Growth per sq. cm. of surface area in millions of organisms.	Growth per cm. of circumference in millions of organisms.
1. Vertical ..	150 × 18	10	2·98	6·12	3,400	340	1,141	556
2. Slanting	150 × 18	10	6·45	16·64	6,370	637	987	383

This experiment was repeated twice with similar results It will be seen that though the quantity of broth, the inoculum, and the duration and temperature of

incubation were exactly the same in the two cases, yet the growth in the slanting tube was almost double the growth in the other. The number of organisms per sq. cm. of the surface area is almost the same in the two cases, i.e., 1,140 millions and 990 millions, and the growth appears to be directly proportional to the surface area. But this correlation is only superficial as is shown by the following experiment:—

Ten-c.c. quantities of a batch of nutrient broth were placed in a test-tube and in a small Haffkine flask, and also 20 c.c. of the same broth were placed in another flask of the same size. The surface of the broth was 2·41 sq. cm. in the test-tube, 50·29 sq. cm. in the flask with 10 c.c. of broth, and 60·84 sq. cm. in the flask with 20 c.c. of broth. 0·5 c.c. of a 48-hour broth culture was seeded into each of the three and shaken thoroughly to mix the inoculum with the broth and incubated at 25°C. for 48 hours. The results obtained are given in Table II :—

TABLE II.

Effect of surface area and its circumference on the growth of Past. pestis *in nutrient broth.*

Ten c.c. of nutrient broth were placed in a test-tube, and 10 c.c. and 20 c.c. of the same nutrient broth were placed in two small Haffkine flasks of the same size. All the three were seeded with 0·5 c.c. each of a 48-hour broth culture. They were all placed in a vertical position in an incubator and incubated at 25°C. for 48 hours.

Container.	Position of container.	Quantity of broth, c.c.	Surface area of broth, sq. cm.	Circumference of broth surface. cm.	Total growth in millions of organisms.	Growth per c.c. of broth in millions of organisms.	Growth per sq. cm. of surface area in millions of organisms.	Growth per cm. of circumference in millions of organisms.
1. Test-tube ..	Vertical	10	2·41	5·47	5,070	507	2,104	927
2. Small Haffkine flask.	,,	10	50·29	25·13	25,750	2,575	512	1,024
3. Do. ..	,,	20	60·84	27·65	21,000	1,050	345	760

In this case the ratios of surface areas of the broth in the two flasks to the surface area of the broth in the test-tube are 25 to 1 and 21 to 1 respectively, while the ratios of the growths are 4 to 1 and 5 to 1, clearly showing that the growths in the three containers are not proportional to the

surface areas. Nor have the growths any relationship to the quantities of the broth, because 10 c.c. of the broth in the test-tube yielded only about 1/5th of the growth in the same quantity of the broth in the first flask. And the total growths in the two flasks are about the same, although the quantity of broths in one flask is twice as much as in the other. It also follows that the depth of the medium also has no effect on the growth. It is thus clear that neither the surface area nor the quantity of the broth nor the depth of the medium affects the total growth, but the data given in Table II disclose another possible relationship. The growths are directly proportional to the length of the circumferences of the surface areas of the broth in the three containers. Each centimetre length of the circumference in the three containers yields about equal number of organisms, i.e , 927, 1,024, and 760 millions. Clearly the total amount of growth is conditioned by the length of the circumference of the surface area of nutrient broth. This relationship was masked in the experiment described in Table I because the ratio of the two surface areas of the broth and the ratio of the circumferences of the broth surfaces in the two test-tubes were of the same order. The difference between growths noted with reference to the length of the circumference of the broth surface is well within the experimental error of the method of determining the number of organisms.

The effect of the surface area of the nutrient broth or its circumference has been stressed because nutrient-broth tubes are usually placed in wire baskets of various sizes for keeping them in incubators, and the tubes thus usually assume any undetermined inclination to the horizontal. This circumstance produces great differences in the resultant growths. For obtaining relatively equal amounts of growth, tubes of exactly the same internal diameter should be used and they must be kept in a vertical position while undergoing incubation.

The same circumstance, i.e , the growth of the organism on the surface of the liquid medium or its periphery, imposes another restriction. Irregular amounts of growth were obtained over a period of time during a series of determinations carried out to compare the growth of *Past. pestis* at different temperatures of incubation, but a careful examination of the data showed that the growths were always more when the tubes were put in the incubator on Saturday mornings and taken out on Monday mornings. This happened to be the time of the week when the incubator was not used by other workers for putting in their cultures. During this period of the week the tubes were allowed to incubate absolutely undisturbed. It thus transpired that the slight jars resulting from the frequent opening and shutting of the incubator door made some of the growing organisms lose their hold on the surface and drop into the body of the medium, thus ceasing to multiply further. Since then, a special low temperature incubator has been installed on the ground floor of the Institute and used exclusively for this purpose.

Thus, if the same quantity of the same batch of nutrient broth is placed in tubes of the same internal diameter and exactly the same amount of inoculum of the same strain of the organism is used and the tubes are placed in the incubator in an absolutely vertical position and incubated at a given temperature for the same period of time absolutely undisturbed, the growths obtained on two or more different occasions when measured by the method of colony counts described here agree

very closely. Three growths in one batch of broth gave the following results : $6\cdot15 \times 10^8$, $5\cdot27 \times 10^8$, and 6×10^8 organisms per c.c. and with another batch of broth $3\cdot58 \times 10^8$, $3\cdot37 \times 10^8$, and $3\cdot69 \times 10^8$ organisms per c.c.

The second angle from which the accuracy of the method must be assessed is : How do the counts obtained by this method compare with the number of organisms actually present in a given broth culture or suspension of plague organism ? This assessment can be done either (1) by comparing the counts obtained by the colony-count method with those obtained by direct microscopic observation, (2) by measuring the biological response to infection, or (3) by statistical evaluation.

Counts of numbers by direct microscopic observation present certain difficulties. The organisms do not settle down rapidly in the counting chamber and show considerable Brownian motion. Further, by microscopic observation both viable and dead organisms are counted. Subject to these disadvantages counts were carried out on three different broth suspensions by both the colony-count method and under the microscope with a Petroff-Hausser Bacteria Counting Chamber.

The following results were obtained :—

Suspension.	Colony count, organisms per c.c. in millions.	Microscopical count, organisms per c.c. in millions.
1st	52	56
2nd	78	85
3rd	79	95

The direct counts are higher than the colony counts in all cases, maximum excess being 20 per cent. Direct counts under the microscope do not distinguish viable organism from non-viable ones, and are likely to be higher than colony counts by plating as the latter method enumerates only viable organism. In any case the correspondence of results is fairly close.

The accuracy of the colony counts was also tested by measuring the biological response of a highly susceptible animal to infection. As will be shown in a later paper the strain of white mouse inbred at the Haffkine Institute has been found to give extremely satisfactory results with doses which at most represent a few units. If progressive tenth dilutions of a suspension are made

and the number of organisms enumerated by the colony-count method described, a dilution will be reached in which no organism will be present in the volume of the dilution elected for injection per animal. Then, if such a volume of such a dilution is given to a number of animals of the selected susceptible strain, no mortality should occur if the colony counts agreed with the number of organisms actually present in the volume of the selected dilution injected per animal.

Progressive tenth dilutions in saline of 48-hour broth growths of two virulent strains were made in 10-c.c. quantities and were administered to highly susceptible white mice.

The results are given in Tables III and IV :—

TABLE III.

Verification of colony counts by animal experiments.

Virulent plague strain I.—48-hour growth at 25°C. in nutrient broth gave a colony count of $1\cdot6 \times 10^8$ organisms per c.c. Progressive tenth dilutions in saline were made and 0·5 c.c. of dilutions 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} were administered subcutaneously per mouse.

Dilution.	Number of organisms per 0·5 c.c. of dilution used as the infective dose per animal.	Number of mice infected.	Number of deaths.	Average number of days elapsing between infection and death.
10^{-6}	80	5	5	4·8
10^{-7}	8	5	5	7·2
10^{-8}	1*	5	0	..
10^{-9}	?	5	0	..

* This dilution was made by adding 1 c.c. of the previous dilutions to 9 c.c. of saline and would thus contain 16 organisms in 10 c.c. These 16 organisms are not likely to be so uniformly distributed in 10 c.c. as to contain one organism per 0·5 c.c. of the infective dose used.

? 10 c.c. of this dilution will contain theoretically less than two organisms.

TABLE IV.

Verification of colony counts by animal experiments.

Virulent plague strain 39/B.—48-hour broth growth with a colony count of $1·7 \times 10^8$ organisms per c.c., progressive tenth dilutions in saline were made, and 0·5 c.c. of dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} injected per mouse.

Dilution.	Number of organisms per 0·5 c.c. of dilution used as the infective dose per animal.	Number of mice infected.	Number of deaths.	Average number of days elapsing between infection and death.
10^{-1}	8,500.000	5	5	2·0
10^{-2}	850,000	5	5	2·4
10^{-3}	85,000	5	5	3·4
10^{-4}	8,500	5	5	3·1
10^{-5}	850	5	5	3·6
10^{-6}	85	5	5	4·2
10^{-7}	9	5	5	5·0
10^{-8}	1*	5	2	7·5

*Here again the same phenomenon is repeated as in the last experiment. Total number of organisms in 10 c.c. of the last dilution contained 18 organisms according to colony counts. Since the chances of this small number of organisms being uniformly distributed in 10 c.c. saline are small when 0·5 c.c. of this dilution was given per mouse, only two animals died.

The animal experiments show that the colony counts agree closely with the number of organisms actually present in a dilution.

The accuracy of the colony counts made by this method, when checked by the statistical method suggested by Fisher (1936), is of a high order. Agreement of the observed distribution with the Poisson distribution affords in the dilution method of counting a test of the suitability of the technique and medium. If the technique of dilution affords a perfectly random distribution of bacteria, the medium used for plating is entirely suitable and the organism can develop on the plates without mutual interference, then the observations will agree with the

Poisson distribution. This test was applied to 82 sets of four parallel plates each, which had been employed for certain measurements.

For each set of these parallel plates the value of χ^2 was calculated by applying the formula

$$\chi^2 = \frac{S(x - \bar{x})^2}{\bar{x}}$$

in which $x_1, x_2 \ldots \ldots \ldots x_n$ are the number of colonies on each individual plate and $\bar{x}$ the mean of these numbers. For true samples of a Poisson series, χ^2 calculated in this way will be distributed in a known manner (Fisher, *loc. cit.*). For a set of four plates χ^2 will vary between 0 and 11·341. The expected and observed distributions of χ^2 in this particular observation are given in Table V. It will be seen that considering that 82 sets of only four parallel plates were used, the agreement between the expected and observed distribution is fairly good, showing that the technique of colony counts described is suitable. A large amount of data of counts employing eight parallel plates for each count is being collected and will be presented in a later communication.

TABLE V.

Test of agreement with Poisson series. For bacterial colony counts.

82 sets of four parallel plates.

χ^2	Expected frequency percentage.	Observed frequency percentage.
0–0·115 ..	1	..
0·115–0·185 ..	1	..
0·185–0·352 ..	3	..
0·352–0·584 ..	5	..
0·584–1·005 ..	10	7·41
1·005–1·424 ..	10	12·35
1·424–2·366 ..	20	17·28
2·366–3·665 ..	20	28·40
3·665–4·642 ..	10	13·58
4·642–6·251 ..	10	8·64
6·251–7·815 ..	5	6·17
7·815–9·837 ..	3	3·70
9·837–11·341 ..	1	2·47
Above 11·341 ..	1	..

SUMMARY.

A method (employing blood-agar surfaces and 37·5°C., the temperature of incubation) is described for counting the number of viable plague organisms present in broth cultures or suspensions. It consists in making progressive tenth dilutions of the broth culture or suspension and spreading by a special pipette 0·05 c.c. of a suitable dilution on 40 sq. cm. surface of blood agar in sets of eight Legroux tubes. A suitable dilution is one which would give 10 to 40 colonies after incubation at 37·5°C. For a 48-hour broth culture incubated at 27°C. a 10^{-6} dilution usually gives the required number of colonies. The counts obtained by this method are shown to agree closely with the actual number of organisms present in a dilution counted by direct enumeration under the microscope and by the biological response of a highly susceptible animal to infection; the method is shown to be statistically valid.

It is shown that a 48-hour growth of the plague bacillus in a liquid medium bears no relation to the total quantity as such of the medium nor to its surface area, but is directly proportional to the circumference of its surface area. This fact imposes certain restrictions when attempts are made to compare growths obtained on two or more different occasions by using equal quantities of the same batch of nutrient broth and the same amount of inoculum of the same strain of the organism. Successive growths give very close results by this method of counting, if the same quantity of the same batch of nutrient broth is placed in tubes of the same internal diameter, and exactly the same amount of the inoculum of the same strain of the organism is used, and the tubes are placed in the incubator in a vertical position and incubated in an undisturbed state free from jars, at a given temperature for the same period of time.

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EXPERIMENTAL STUDIES IN PLAGUE.

Part IV.

EXPERIMENTAL ANIMAL OF CHOICE FOR PLAGUE WORK.

BY

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PREVIOUS workers have used a wide variety of experimental animals, monkeys, rabbits, guinea-pigs, white rats, wild house rats, and white mice, but preference lay with the guinea-pig as the most susceptible animal although for convenience wild house rats (*Rattus rattus*) and white rats were also freely used. Petrie (1929), summing up the reported work, says, 'White rats and guinea-pigs possess an almost uniform susceptibility to plague, mice are less susceptible and rabbits are least susceptible to the infection'. Otten (1933) more recently came to the conclusion that the guinea-pig was the animal most susceptible to plague; after the guinea-pig he placed the wild rat, the white mouse, and the white rat in their order of susceptibility. He says, 'Obviously wild rats and guinea-pigs largely differ as to their susceptibility to plague infections; infected with 20 to 50 bacilli only 35 per cent of the wild rats were killed, whereas the mortality rate of guinea-pigs was almost 100 per cent; with 2 to 5 bacilli all wild rats resisted and still 85 per cent of guinea-pigs died'. His experimental work further showed that a single bacillus of a highly virulent strain was enough to kill a guinea-pig. He also concluded that though the white mouse seemed to be almost as susceptible as the wild rat, the susceptibility of the mouse was not specific, and was not constant. He found that white mice infected with a very large infective dose tended to escape death. He not only found the white rat to be the least susceptible but also not constant in this respect.

Over a long period of time the wild house rat (*Rattus rattus*) had been preferred as the experimental animal for plague work at the Haffkine Institute. As the Bombay wild house rat had become resistant to plague, wild house rat was imported monthly for this purpose from Madras city which had not suffered from any serious plague epidemics during the present pandemic.

As stated in the Introduction to this series of papers (*see* Part I) the method hastily improvised in 1928 to check up the routine method (Naidu *et al.*, 1926) in use at the time for measuring the protective power of Haffkine vaccine also employed the wild house rat (Madras) as the test animal. As determinations on the same batches of the vaccine over and over again with various changes in the preparation and nature of the infective dose with the use of the wild house rat as the experimental animal gave very wide variations in results it appeared possible that the wild rat was unsuitable for the purpose. This work also showed that plague-broth cultures could with advantage be used for inducing test infection. The British Plague Commission (1912) had found broth cultures of little use for this purpose. Writing on ' the immunity of the wild house rat in India ' the Commission stated, ' again it was a matter of no small difficulty to find some means of regulating the dose of plague bacilli injected so as to maintain an even dose in a long series of experiments ; two variable factors had to be regulated, the virulence of the organism and the number used in each dose. We soon learnt that *broth cultures were of little or no use for our purpose*, and after a prolonged series of trials of different methods we adopted the method of spleen emulsions '. Both the difficulties encountered by the British Plague Commission in the use of broth cultures for the preparation of a suitable test infective dose have been got over ; the number of organisms contained in an infective dose can be accurately counted by the method described in Part III of this paper (Sokhey, 1939b), and the virulence of the organisms can be maintained unaltered for years by the method briefly described elsewhere (Sokhey, 1936) and to be fully reported in Part VI of this communication. These methods permit of the use of broth cultures for the preparation of a truly constant test infective dose, constant both as regards the virulence of the organisms and the numbers used. Such an infective dose compares very favourably with the spleen-emulsion method the constancy of which is largely deceptive (Sokhey, 1939a). Having thus devised the means for preparing constant infective doses the question of the suitable test animal was then investigated and the results are reported here.

Test infective doses were prepared from 48-hour broth cultures grown at 25°C. Three different highly virulent strains of *Past. pestis* were employed. Progressive tenth dilutions in saline were made from 48-hour broth cultures and the numbers of organisms contained in them determined by colony-count method by plating (Sokhey, 1939b). Suitable quantities of these dilutions containing a known number of plague organisms were selected and administered subcutaneously to batches of selected species of animals with a view to determine the minimum lethal infective dose for each species. This was further particularized by the time elapsing between infection and death. Animals were observed for a period of 25 days after the induction of infection. Those dying within this period were dissected and examined for evidence of plague by microscopical examination of smears, and failing that by cultural methods. Animals which survived were

killed at the end of this period of observation of 25 days and similarly examined. Animals which died during this period always showed plague bacilli in spleen and liver smears, but no plague organisms were ever seen in the smears of these organs from those animals which survived the period of 25 days. The results are given in Tables I to X :—

TABLE I.

Relative susceptibility of various laboratory animals to plague infection.

Past. pestis strain I. Experiment 1.

Animal.	Average weight in grammes.	NUMBER OF VIABLE ORGANISMS PER ANIMAL GIVEN SUBCUTANEOUSLY.			
		10	95	955	95,500
White mouse (Haffkine Inst.) ..	25	5/5*	5/5	..	..
		(5·8)†	(5·2)	..	..
Wild house rat (Madras) ..	82	1/5	3/5	5/5	..
		(8)	(10)	(5·6)	..
White rat	213	0/5	5/5	5/5	5/5
			(7·4)	(5·8)	(3·6)
Guinea-pig	545	1/5	5/5	2/5	..
		(13)	(11·6)	(9·0)	..

* The numerators give the number of deaths and the denominators the total number of animals used.

† The figures in brackets are the average number of days elapsing between infection and death.

TABLE II.

Relative susceptibility of various laboratory animals to plague infection.

Past. pestis strain I.   Experiment 2.

Animal.	Average weight in grammes.	NUMBER OF VIABLE ORGANISMS PER ANIMAL GIVEN SUBCUTANEOUSLY.			
		8	40	83	830
White mouse (Haffkine Inst.) ..	23	4/5*	5/5	..	..
		(5·7)†	(4·8)	..	..
Wild house rat (Madras) ..	110	2/5	2/5	2/5	3/5
		(10)	(10)	(9·5)	(5)
White rat	126	2/5	5/5	3/5	3/5
		(5·5)	(5·8)	(4·4)	(5)
Guinea-pig	395	3/5	4/5	3/5	1/5
		(13)	(9·5)	(8·3)	(10)

TABLE III.

Relative susceptibility of various laboratory animals to plague infection.

Past. pestis strain I.   Experiment 3.

Animal.	Average weight in grammes.	NUMBER OF VIABLE ORGANISMS PER ANIMAL GIVEN SUBCUTANEOUSLY.				
		8	80	800	8,000	800,000
White mouse (Haffkine Inst.) ..	24	5/5*	5/5	..	..	..
		(7·2)†	(4·8)	..	..	..
Wild house rat (Madras) ..	112	2/5	5/5	5/5	3/5	..
		(7)	(5·8)	(8·6)	(5·7)	..
Guinea-pig	436	3/5	3/5	3/5	..	4/5
		(14·7)	(10)	(10)	..	(10)

     * The numerators give the number of deaths and the denominators the total number of animals used.

     † The figures in brackets are the average number of days elapsing between infection and death.

TABLE IV.

Relative susceptibility of various laboratory animals to plague infection.

Past. pestis strain I. Experiment 4.

Animal.	Average weight in grammes.	NUMBER OF VIABLE ORGANISMS PER ANIMAL GIVEN SUBCUTANEOUSLY.			
		10	100	1,000	10,000
White mouse (Haffkine Inst.)	24	4/5*	10/10	..	..
		(6·2)†	(5·8)	..	..
Wild house rat (Madras) ..	131	1/5	2/5	3/5	3/5
		(9)	(5·5)	(6·3)	(4·6)
White rat	177	3/5	3/5	4/5	3/5
		(6)	(8·6)	(6·2)	(5·6)

TABLE V.

Relative susceptibility of various laboratory animals to plague infection.

Past. pestis strain 179/5H.

Animal. .	Average weight in grammes.	NUMBER OF VIABLE ORGANISMS PER ANIMAL GIVEN SUBCUTANEOUSLY.			
		6	58	585	5,850
White mouse (Haffkine Inst.)	25	5/5*	5/5	..	..
		(5)†	(4·4)	..	..
Wild house rat (Madras) ..	139	4/5	4/5	..	..
		(8)	(7·3)	..	..
White rat	206	2/5	2/5	3/5	3/5
		(5·5)	(4·5)	(3·3)	(2·7)
Guinea-pig	617	5/5	4/5	4/5	..
		(9·4)	(10)	(7·3)	..

* The numerators give the number of deaths and the denominators the total number of animals used.

† The figures in brackets are the average number of days elapsing between infection and death.

TABLE VI.

Relative susceptibility of various laboratory animals to plague infection.

Past. pestis strain 39/B.

Animal.	Average weight in grammes.	NUMBER OF VIABLE ORGANISMS PER ANIMAL GIVEN SUBCUTANEOUSLY.								
		1	9	85	850	8,500	85,000	850,000	8,500,000	85,000,000
White mouse (Haffkine Inst.).	22	2/5* (7·5)†	5/5 (5·0)	5/5 (4·2)	5/5 (3·6)	5/5 (3·1)	5/5 (3·4)	5/5 (2·4)	5/5 (2)	..
Wild house rat (Madras)	88	..	1/5 (9)	4/5 (5·7)	2/5 (4·5)	3/5 (5·9)	5/5 (4·6)	5/5 (2·2)	5/5 (2·0)	..
White rat ..	235	..	0/5	4/5 (8·2)	5/5 (4·6)	5/5 (4·6)	5/5 (2·6)	5/5 (2·4)	5/5 (2·2)	..
Guinea-pig ..	590	..	..	3/5 (13·7)	1/5 (11)	3/5 (10·0)	4/5 (10·5)	5/5 (10·0)	4/5 (4·2)	5/5 (0·5)

* The numerators give the number of deaths and the denominators the total number of animals used.
† The figures in brackets are the average number of days elapsing between infection and death.

TABLE VII.

Relative susceptibility of various laboratory animals to plague infection.

Past. pestis strain I.

Animal.	Average weight in grammes.	NUMBER OF VIABLE ORGANISMS PER ANIMAL GIVEN SUBCUTANEOUSLY.									
		11	112	755	7,325	14,700	147,000	567,000	1,470,000	2,122,000	15,033,000
White mouse (Haffkine Inst.).	23	23/24* (6·7)†	24/24 (5·2)	..	..	..	..	..	..	..	..
Wild house rat (Madras)	126	..	..	3/5 (7·0)	10/20 (5·5)	10/10 (4·9)	10/10 (4·7)	..	7/13 (9·3)	..	..
White rat ..	237	..	..	6/10 (5·7)	12/20 (4·5)	18/20 (4·3)	..	10/10 (2·2)	..	..	..
Guinea-pig ..	435	..	..	..	..	..	..	20/30 (9·1)	..	6/10 (9·1)	20/30 (9·4)

* The numerators give the number of deaths and the denominators the number of animals used.

† The figures in brackets are the average number of days elapsing between infection and death.

TABLE VIII.

Susceptibility of white mice from Pasteur Institute, Bandoeng, Batavia, to plague infection.

Past. pestis strain 39/B. Experiment 1.

Animal.	Average weight in grammes.	Number of viable organisms per animal given subcutaneously.							
		?	4	44	440	4,400	44,000	440,000	44,000,000
White mouse (Pasteur Institute, Bandoeng).	22	0/5*	3/5 (8·0)†	5/5 (5·4)	5/5 (5·2)	5/5 (2·6)	5/5 (3·4)	5/5 (3·4)	5/5 (2·2)

TABLE IX.

Susceptibility of white mice from Pasteur Institute, Bandoeng, Batavia, to plague infection.

Past. pestis strain 39/B. Experiment 2.

Animal.	Average weight in grammes.	Number of viable organisms per animal given subcutaneously.							
		?	3	35	350	3,500	35,000	350,000	35,000,000
White mouse (Pasteur Institute, Bandoeng).	22	1/5* (5)†	4/5 (6·3)	4/5 (5·7)	5/5 (4·4)	5/5 (3·6)	5/5 (2·4)	5/5 (2·2)	5/5 (3·2)

* The numerators give the number of deaths and the denominators the total number of animals used.
† The figures in brackets are the average number of days elapsing between infection and death.

TABLE X.

Response of Haffkine-Institute-inbred white mouse to plague infection summarized.

Table number.	MINIMUM DOSE PRODUCING APPROXIMATELY 100 PER CENT MORTALITY.		TEN TIMES THE MINIMUM DOSE.	
	Number of organisms.	Average number of days elapsing between infection and death.	Number of organisms.	Average number of days elapsing between infection and death.
I ..	10	5·8	95	5·2
II ..	8	5·7	40*	4·8
III ..	8	7·2	80	4·8
IV ..	10†	6·2	100	5·8
V ..	6	5·0	58	4·4
VI ..	9	5·0	85	4·2
VII ..	11	6·7	112	5·2

* Only five times the first dose.
† One animal escaped death.

In the case of the Haffkine-Institute-inbred white mouse, average weight 23 g., as few as 6 to 12 organisms were enough to produce a 100 per cent mortality. With this dose average number of days elapsing between infection and death varied between 5 and 7·2 days. When this dose was increased tenfold the average duration of life after infection was, however, reduced to 4·2 to 5·8 days. When progressively larger infective doses were used the period elapsing between infection and death was progressively reduced, and with a very big dose, but not so big as to kill by its toxicity, this period was reduced to about two days. It must be stated here that 6 to 12 organisms are not the absolute minimum dose to produce a 100 per cent mortality. In several other experiments not reported here a smaller dose was found to be enough, but to achieve the smaller dose with any degree of certainty, when a few organisms are discontinuously distributed inasmuch as 10 c.c. of fluid, as is the case with this method of preparing the infective dose, is not possible. Thus, when 120 organisms are present in 10 c.c. of a suspension and each animal

is given 0·5 c.c. of this suspension it is not unlikely that some animals receive perhaps no more than one organism. This phenomenon is objectively illustrated when these very high dilutions are plated on blood-agar plates.

It is to be noted that the response to infection of the strain of white mouse from Dr. L. Otten's laboratory, Pasteur Institute, Bandoeng, Batavia, was in no way different to the response of the Haffkine-Institute-inbred white mouse. The peculiar phenomenon noted by Otten (*loc. cit.*) ' that in white mice infected with the more concentrated dilutions escapes occurred ', has not been confirmed. With 10^{-1} to 10^{-3} dilutions of his suspension Otten obtained a mortality of only 90 per cent; in the writer's hands Otten's strain of mouse has behaved in the same way as the Haffkine Institute strain, as all the concentrated ·dilutions (10^{-2} to 10^{-5}) consistently killed 100 per cent of the mice infected.

The response to plague infection of the wild house rat (Madras), the white rat, and the guinea-pig is exceedingly variable. From the experiments reported here it is not possible to say what doses could be depended upon to produce 100 per cent mortality among these animals. In the experiments reported in Tables I to VI small batches of animals had been used, and it left a doubt whether the inconclusiveness of the results was not due to this factor; in Table VII therefore the results of experiments carried out with larger batches of animals have been summarized. These results are no more conclusive than those reported in Tables I to VI. Thus, in the case of the wild house rat (Madras) the 100 per cent fatal dose has varied from: 800 organisms to 85,000 organisms, in the case of the white rat from 40 to 8,500 organisms, and in the case of the guinea-pig from 6 to over 15 millions organisms. These animals were infected with the same strains of *Past. pestis* as were used for infecting the white mouse in which case as few as 10 organisms were sufficient to produce a 100 per cent mortality. The absolutely constant response of the white mouse showed that the strains of *Past. pestis* used were fully virulent. The very variable results in the case of other animals were due to the variable response to plague infection of these animals—at least of the strains of the animals used in these experiments. The wild house rat (*Rattus rattus*) was imported from Madras, a city which has not suffered from plague during the present pandemic. The strain of white rat used was obtained from the Nutrition Laboratory, Coonoor. The guinea-pig used belongs to a strain which has been used in India in the different laboratories over a period of years, and has been in use in the Haffkine Institute for the last 20 years or more.

The results do show that the greater and almost constant susceptibility of the white mouse, both of the Haffkine-Institute-inbred strain and of the Pasteur Institute, Bandoeng strain, is specific and is not conditioned by the smaller body-weight of this animal as compared with the body-weight of the other animals. If the body-weight were the conditioning factor and if 10 organisms were the fatal dose for a mouse of 23 g. weight, doses of 225 organisms, 50 organisms, and 90 organisms would suffice for the guinea-pig (516 g.), wild house rat (110 g.), and white rat (191 g.), respectively. This is not so is conclusively shown by the experiments reported here.

The white mouse, in fact, fulfils all the requirements needed for experimental work. It is the animal of choice for plague work. During the years 1934–1937,

1,180 white mice have been used in batches of 5 or 10 as controls for experiments for measuring active immunity produced by plague vaccine or for measuring the curative power of antiplague sera. The different batches received two to ten times the minimum lethal dose, i.e., 22 to 212 organisms per animal. These experiments have shown the same constancy of the white mouse to plague infection both as regards the fatal termination and duration of life after infection as the experiments reported above. Of 1,180 animals used 17 escaped death, giving a percentage escape of 1·5 and duration of life after infection varied between 4·0 days and 7·0 days (except in the case of one batch of 5 mice for which the average duration of life after infection was 8·8 days).

White mice between the ages of $2\frac{1}{2}$ months and 6 months respond equally well, but animals between the ages of $2\frac{1}{2}$ months and 4 months are preferred. The animals usually used for the work weigh between 22 g. and 28 g., the females being nearer the lower limit. Males and females do not show any difference in their response to plague infection, but members of a particular experimental batch of five mice are always made up of animals of one sex.

The breeding of white mice under the climatic conditions of Bombay presents certain difficulties, but methods have been developed which enable a healthy stock to be bred and maintained. It is possible that experience in this connection may be of value to other workers in the tropics, that a note on ' Breeding and Care of the White Mouse ' is attached as an *Appendix* to this paper. Attention is especially drawn to the cage for housing mice under experiment, which has been designed by the author for use in the tropics. After a great deal of experience, it was realized that in hot climates mice require considerably more space than is provided in the usual cages in use in America and Europe. The cage is so designed that its component parts come apart easily to permit of thorough cleaning and is illustrated in Plate XVII, figs. 1 and 2, of the *Appendix*.

Summary.

Experimental evidence has been produced to show that the white mouse (Haffkine-Institute-inbred strain) is the experimental animal of choice for plague work. It is very highly susceptible to plague infection and the response of individual members of this species is constant. As few as 10 organisms, perhaps even less, per animal are enough to produce a 100 per cent mortality.

It is further shown that the white rat, the wild house rat, and the guinea-pig are much less susceptible to plague infection. The response of the individual members of these species is so variable that it is difficult to fix a hundred per cent fatal dose for them. About 10 organisms per animal produce a 100 per cent mortality in the white mouse, whereas 800 to 85,000 organisms were needed in the case of the wild house rat, 40 to 8,500 organisms for the white rat, and 6 to over 15 millions organisms in the case of the guinea-pig. It is also pointed out that the response of the white mouse is specific and is not conditioned by the smallness of its body-weight.

, A note on ' Breeding and Care of the White Mouse ' is attached as an *Appendix* to this paper.

ACKNOWLEDGMENT.

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PLATE XVI.
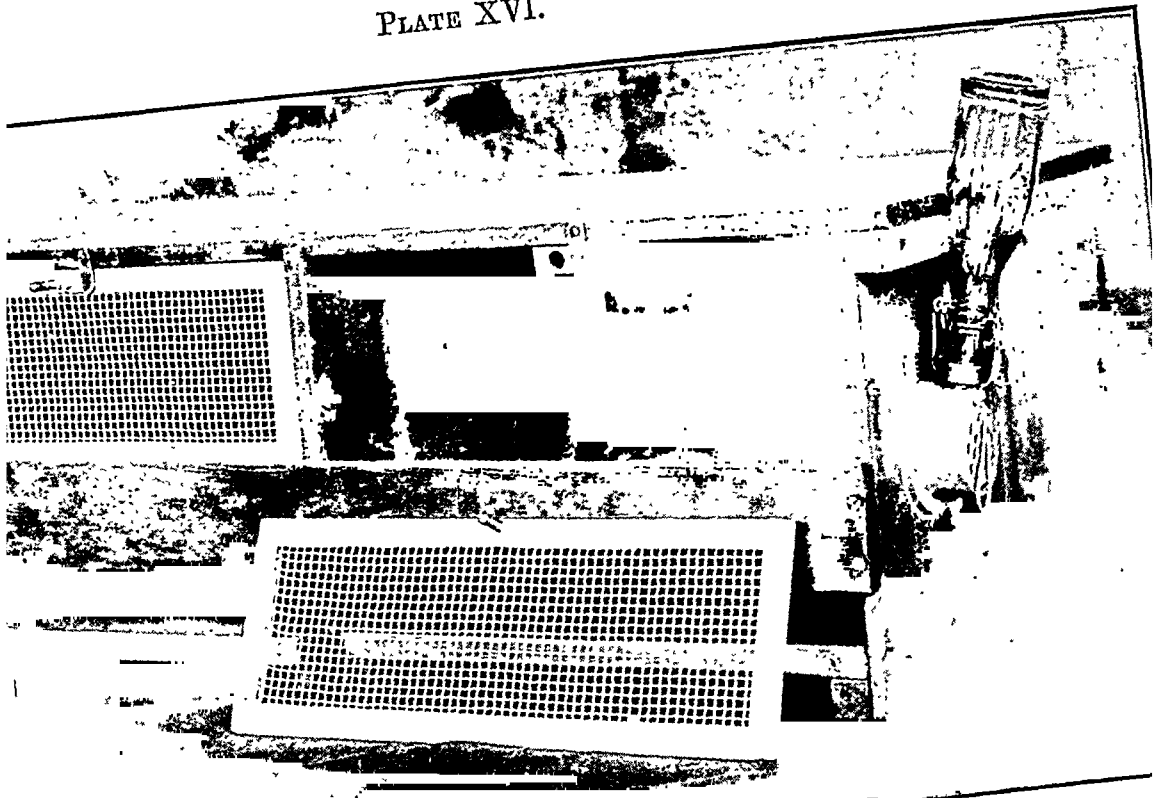
Fig. 1.—The Wistar Institute 'dormer cage'.

Fig. 2.—The nest cage.

PLATE XVII.
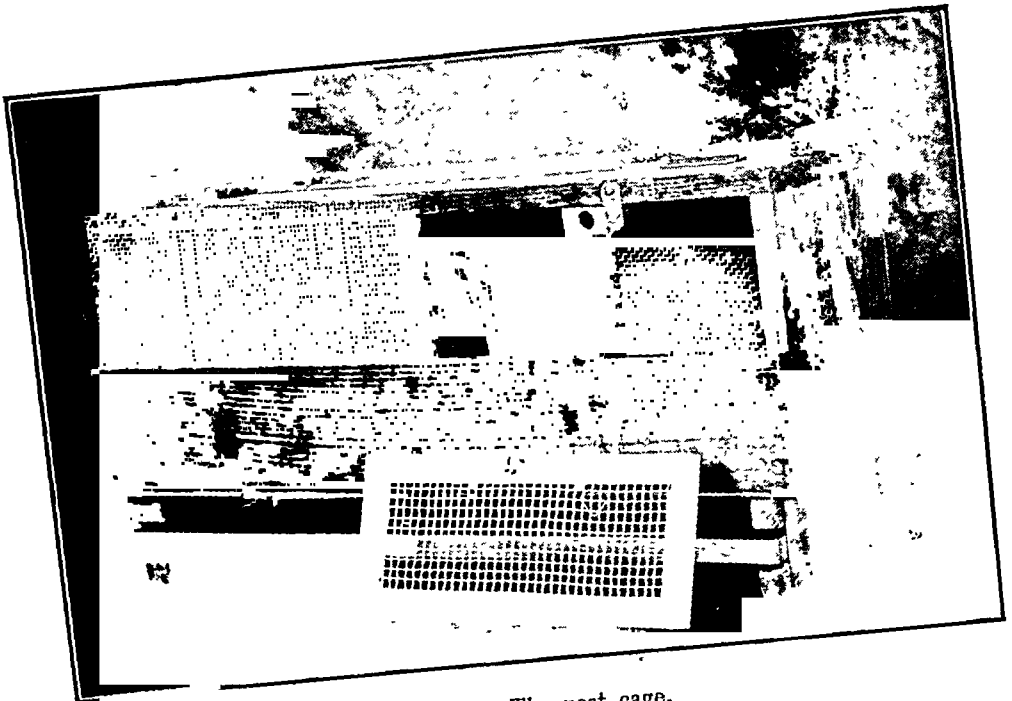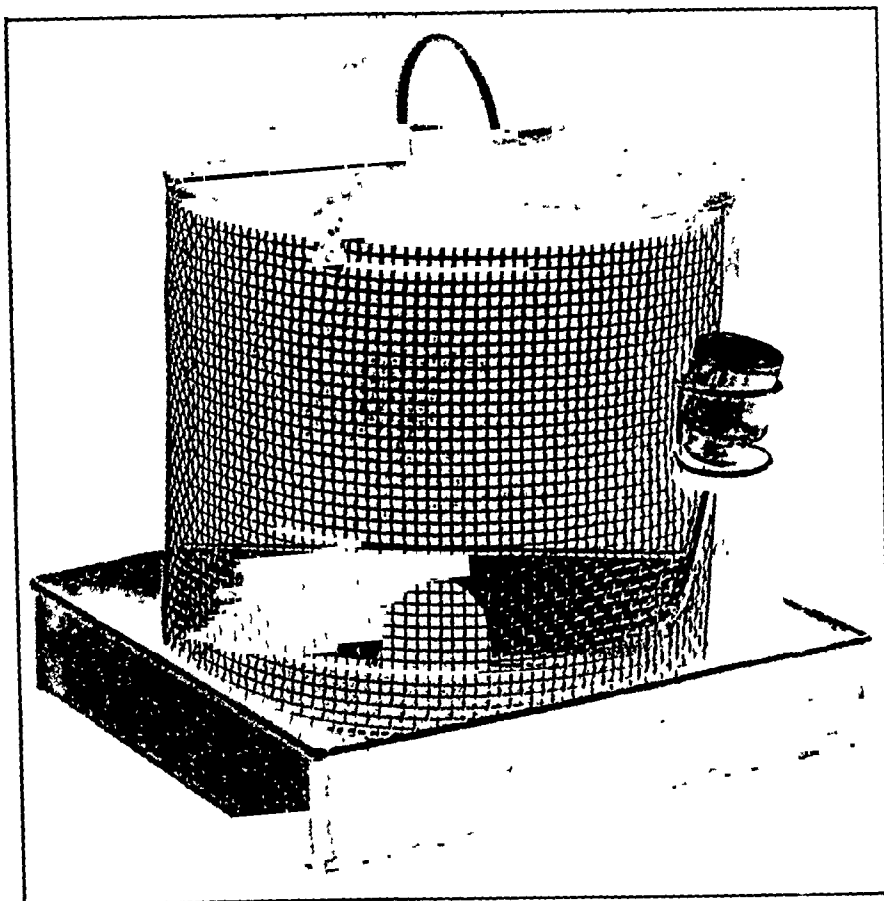
Fig. 1.—The cage for experimental animals.
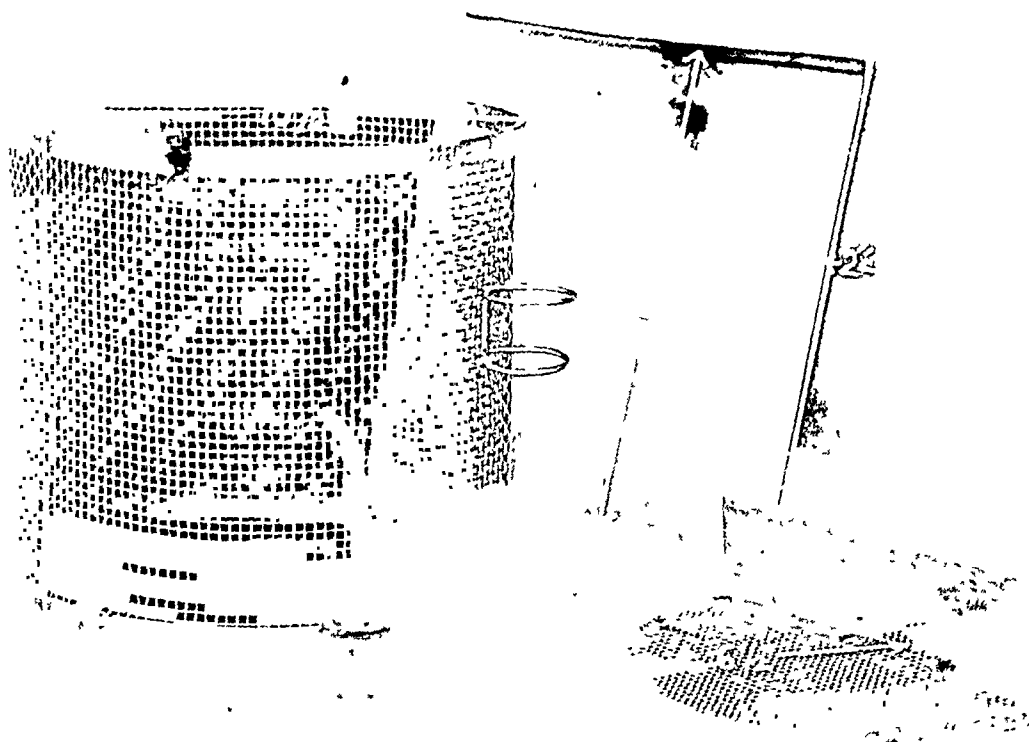
Fig. 2.—The same cage dismantled.

APPENDIX.

BREEDING AND CARE OF THE WHITE MOUSE.

AT the Haffkine Institute a population of about 4,000 white mice divided into two separate colonies is maintained, and from 800 to 1,000 mice are bred per month. As success has been obtained in keeping these colonies free from any extraneous infection, a few notes are appended so that they may be of help to other workers in the tropics.

A. *Cages.*—Three types of cages are used.

(1) *The Wistar Institute ' dormer cage '* is used for housing stock animals and for mating. The cage is illustrated in Plate XVI, fig. 1, and is fully described in ' Breeding and Care of the Albino Rat for Research Purposes ' by Milton, Greenman and Louise Duhring: Philadelphia, the Wistar Institute of Anatomy and Biology; 1931. Thirty adult mice are housed in each dormer cage. For mating, 10 females and one male are put in one dormer cage.

(2) *Nest cage.*—This is a small modified ' dormer cage ', measuring $21\frac{1}{2}$ inches long, $8\frac{1}{2}$ inches high, and $7\frac{1}{2}$ inches wide. Only one compartment is provided with a door, the other compartment is closed in by a fixed galvanized wire cloth, $\frac{1}{8}$ inch mesh. The top of the cage is hinged to permit the cleaning of the compartments. Instead of the drinking fountain which goes with the large dormer cage a simpler form of drinking tube is fixed inside the cage in the compartment fitted with the door. The cage is illustrated in Plate XVI, fig 2. Two pregnant females are put in one of these nest cages.

(3) *Cage for experimental animals.*—This is a cylindrical cage made of galvanized wire cloth (No. 18 wire $\frac{1}{5}$ inch mesh), $10\frac{1}{4}$ inches high, and $10\frac{1}{2}$ inches in diameter. The circular floor of screen of the cage is detachable and is made of galvanized wire cloth, and has a metal rim. This circular floor plate is made of wire cloth No. 20 wire $\frac{1}{8}$ inch mesh) and is supported on four metal supports which project inwards from the bottom rim of the cylinder and also downwards to form support for the cage itself. The floor plate is divided into two unequal portions with a vertical partition $2\frac{1}{4}$ inches high. In the middle of the partition there is an opening. Food is placed on one side of the partition and bedding on the other. The lid of the cage is made of galvanized sheet and fits snugly on the upper rim of the cylinder and is held in place by a piece of galvanized wire bent in the middle to form a handle. The whole cage stands in a rectangular tray. The cage is provided with a drinking fountain and is illustrated in Plate XVII, figs. 1 and 2.

For experimental purposes 5 white mice are housed in one of these cages. Each batch of 5 mice consists of animals of the same sex. This cage is well ventilated and provides enough room for exercise.

B. *Bedding.*—Paper shavings obtained from a printing press are used as bedding in all the cages described. The paper shavings are steam sterilized before use,

(353)

C. *Cleaning of cages.*—All cages in use are cleaned every day ; the mice are taken out in special receptacles, cages manually cleaned and wiped with a dilute antiseptic lotion. Bedding is changed every second day, except in nest cages in which it is not changed for seven days after the birth of the young.

All cages are steam sterilized at least once a month.

D. *Food and feeding.*—Mice receive whole milk, a crushed cereal (oats, barley, or popped corn), green leaves (lettuce, radish tops, turnip tops, carrot tops), carrots, cod-liver oil, whole-meal wheat bread, and eggs, and McCollum-Simmonds salt mixture No. 185 is given to pregnant and nursing females. Cod-liver oil and salt mixture are added to milk. Fresh food is put in cages immediately they have been cleaned in the morning and cages are again examined in the evening and more food put in cages wherever considered necessary. Milk is boiled before serving and cereals are exposed to the sun. Vegetables are carefully washed in clean water and then soaked in potassium permanganate solution, and washed in water again.

EXPERIMENTAL STUDIES IN PLAGUE.

Part V.

A METHOD FOR MEASURING THE VIRULENCE OF PLAGUE CULTURES.

BY

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THE problem of measurement of the virulence of plague organisms consists in the determination of the smallest number of organisms of a given culture, which when administered in a specified manner will kill about 100 per cent individuals of especially selected species, this to be further particularized by the average duration of life after infection. These requirements demand the ability to be able to enumerate the number of organisms in the infective dose and the availability of a highly and uniformly susceptible animal. In the absence of a method for measuring the number of organisms contained in an infective dose the previous workers had to resort to odd devices. Kolle and Martini (1902) emulsified 1/5th of a loopful of a solid growth in 0·2 c.c. of broth and rubbed it on a carefully shaved and abraded area of standard size on a guinea-pig's abdomen. Later, Kolle and Krumbein (1909, quoted by Pollitzer, 1936) employed a syringe needle of standard calibre which was dipped in the bacterial suspension of definite opacity and then introduced it under the skin near the root of the tail of a rat. Rowland (1910) worked with a syringe so arranged that one turn of a screw caused delivery of 0·1 c.c. of the culture to be tested, and the culture was given subcutaneously. In the Haffkine Institute the standard method was to rub into a shaved and abraded patch of skin of a house rat a loopful or so of the culture under test. These were indeed very rough and ready tests and evidently did not achieve their end. Petrie (1929) writing about the time the present work was commenced stated, 'there is no standard method of testing for virulence' of the plague organism.

With the availability of an accurate method of enumerating plague organisms present in a broth culture or a suspension (Sokhey, 1939a) and the availability of a highly and uniformly susceptible laboratory test animal (the Haffkine-Institute-inbred white mouse) (Sokhey, 1939b), the problem of measuring the virulence of the organism becomes a simple matter. It means the determination of the smallest number of organisms which when given subcutaneously to a batch of

Haffkine-Institute-inbred white mice will kill approximately 100 per cent of them. The relative virulence of different strains could be further particularized by the average number of days elapsing between infection and death even though the minimum infective dose producing a 100 per cent mortality may have the same number of organisms. Obviously the larger the number of white mice infected for each test the better, but experience shows that a batch of 5 mice for each dilution works quite well.

Determination of virulence is carried out in the following manner: The culture of which it is desired to test the virulence is usually obtained on an agar slope. A 2-mm. loopful of this growth is inoculated into 10 c.c. of nutrient broth in a test-tube. The growth taken on the loop is carefully mixed with broth to obtain a tolerably uniform suspension. This is then incubated at 25°C. to 27°C. for 48 hours, and 0·5 c.c. of this growth is then added to a fresh tube containing 9·5 c.c. of nutrient broth. The purity of this sub-culture is tested by Gore's method (Taylor, 1933). The second sub-culture is incubated at 25°C. to 27°C. for 48 hours. During this period of incubation the tube is kept in a vertical position and the growth is allowed to proceed in an undisturbed state free from jolts or jars. At the termination of the 48-hour period of incubation of the second sub-culture, the tube is removed from the incubator, and dilutions made as described previously (Sokhey, 1939c). Three or more of these dilutions are selected, which are judged either by a preliminary test or otherwise to contain a fatal dose in 0·2 c.c. of the dilution. The number of organisms contained in these dilutions are counted by the method described previously (Sokhey, 1939a). 0·2 c.c. of the selected dilutions is administered subcutaneously per animal in batches of 5 or 10 mice. A tuberculin syringe (0·01 graduations) with a 27-G needle is used for the purpose. During the earlier part of the work a dose of 0·5 c.c. per animal was used, but now a dose of 0·2 c.c. is found more suitable, as it is less likely to escape through the puncture. In the case of highly virulent strains 0·2 c.c. of a 10^{-7} dilution of a 48-hour broth growth per animal contains the minimal lethal dose. This dilution as well as the next lower (10^{-6}) and the next higher dilutions (10^{-8}) are used. The second broth sub-culture incubated at 27°C. for 48 hours has approximately between 3×10^8 and 6×10^8 organisms per c.c. and 0·2 c.c. of a 10^{-7} dilution would contain 6 to 12 organisms.

The animals after being infected are kept in an air-conditioned room with a constant temperature of 24°C. and a relative humidity of 55 to 60 per cent. Each batch of 5 mice is kept in a separate cage especially designed at the Haffkine Institute for white mice experiments in the tropics (Sokhey, 1939b). The animals are observed for a period of not less than 25 days. Each cage is examined once a day at the same hour and the dead animals are removed. These are dissected and examined, naked-eye signs of plague are noted, and smears are made from spleen and liver and examined under the microscope. In case of doubt, cultures are made for further examination. At the end of the 25 days' period of observation the animals still alive are killed and similarly examined. Animals which die during the period of observation always show the presence of plague bacilli in spleen and liver smears, but no organisms are ever seen in the smears from these organs of those animals which survive the 25 days' period of observation. All

deaths from whatsoever cause are recorded and if an animal dies of an infection or cause other than plague this fact is noted in the protocol, but deaths from other causes are singularly rare, and have been practically absent in this experimental work.

ILLUSTRATIVE EXAMPLES OF VIRULENCE MEASUREMENTS.

A large number of measurements of the virulence of plague organisms has been carried out; a few results are given in Tables I and II to illustrate the type of results the method yields :—

TABLE I.

Measurements of the virulence of some plague cultures.

Culture number.	Number of organisms used as the infective dose per mouse.	Results.	Average duration of life in days between infection and death.
Vimla 10	2 16	5/5* 5/5	12·2 6·2
39/B/10	4 44	5/5 5/5	7·0 5·0
P/25	6 60	5/5 5/5	10·2 5·8
P/5/37	4 40	5/5 5/5	7·8 5·6
P/15/37	8 80	1/5 4/5	7·0 10·0
P/35/37	90 900 9,000	1/5 4/5 5/5	10·0 5·8 5·0
P/40/37	108 1,080	0/5 5/5	.. 6·0
P/50/37	60 600 60,000 600,000	0/5 2/5 5/5 3/5	.. 9·5 10·4 12·7
R. F. M.	7 76 760,000	0/5 0/5 5/5	.. .. 5·6
R/75/37	620,000 62,000,000	2/5 4/5	8·0 6·8

* The numerators give the number of deaths and the denominators the total number of mice infected.

TABLE II.

Measurement of the progressive loss of virulence.

Strain 120/5H.

Culture.	Number of organisms used as the infective dose per mouse.	Results.	Average duration of life in days between infection and death.
Original {	9 93	5/5* 5/5	5·4 5·2
8th sub-culture {	9 92	4/5 4/5	6·0 5·8
16th ,, {	6 62	1/5 5/5	9·0 11·0
24th ,, {	6 62	3/5 5/5	8·3 9·5
32nd ,, {	6 60	4/5 4/5	7·8 8·0
40th ,, {	75 750	0/4 1/4	.. 13
48th ., {	300 3,000	1/5 1/5	8·0 4·0
56th ,, {	12,300 123,000	0/5 0/5	.. ..
64th ,, ..	68,000,000	0/5	..

* The numerators give the number of deaths and the denominators the total number of animals used.

SUSPENSIONS FROM GROWTHS ON SOLID MEDIUM.

It should be stated here that sub-cultures from which the required dilutions for measuring virulence are made should be broth sub-cultures and not agar sub-cultures. Some preliminary experiments show that if suspensions are made from agar sub-cultures, the results are not as constant as with the broth cultures. The reason for this is not clear. The phenomenon is being studied further, but the results of two experiments are given to illustrate the point. From a 48-hour broth culture of a fully virulent strain Ramjee, 10 organisms of which from a broth culture killed 5 out of 5 animals infected, with an average duration of life of five days, two agar slopes, A and B, were seeded and incubated for 48 hours at 27°C. Suspensions in saline were then made from slopes A and B and given to batches of white mice with the results noted in Table III. The result of measurement of the virulence did not agree with that obtained with the broth culture both as regards the total number of deaths and the average duration of life after infection.

TABLE III.

Variable results of agar-growth suspensions.

Agar-slopes A and B were seeded from the same sub-culture in broth of strain Ramjee, and incubated at 27°C. for 48 hours. Suspensions from the two slopes were made in saline separately and given at the same time to batches of mice.

SUSPENSION FROM AGAR-SLOPE A.				SUSPENSION FROM AGAR-SLOPE B.			
Number of organisms in the infective dose.	Number of mice infected.	Deaths.	Average duration of life in days after infection.	Number of organisms in the infective dose.	Number of mice infected.	Deaths.	Average duration of life in days after infection.
19	5	3	7·3	15	5	5	6·0
197	5	5	5·4	155	5	5	4·6

A similar experiment with the virulent strain I gave the results given in Table IV. Here again the results differed both as regards the total number of deaths and the duration of life after infection from those obtained with broth culture. Nine organisms killed 5 out of 5 animals with an average duration of life of six days.

TABLE IV.

Variable results of agar-growth suspensions.

Agar-slopes A and B were seeded from the same sub-culture in broth of virulent strain I and incubated at 27°C. for 48 hours. Suspensions of the two slopes were made in saline separately.

SUSPENSION FROM SLOPE A.				SUSPENSION FROM SLOPE B.			
Number of organisms in the infective dose.	Number of white mice infected.	Deaths.	Average duration of life in days after infection.	Number of organisms in the infective dose.	Number of white mice infected.	Deaths.	Average duration of life in days after infection.
32	5	3	8·7	33	5	4	7·2
320	5	5	6·2	329	5	5	5·2

NEW METHOD COMPARED WITH THE OLD SKIN TEST.

The standard practice at the Haffkine Institute for measuring the virulence of plague cultures formerly was to rub on a shaved and abraded patch on a house rat's abdomen a loopful or so of the culture to be tested. Usually only one rat was used at a time. In Table V the results of comparative measurements by the skin test and the new test are given :—

TABLE V.

Number of culture.	Old test.	New test.	
		Number of organisms given.	Results.
P/5/37	2/2* (4·2)	4	5/5 (7·8)†
		40	5/5 (5·6)
P/15/37	2/2 (4·0)	8	1/5 (7·0)
		80	4/5 (10·0)

* The numerators give the number of deaths and the denominators the total number of animals used.

† The figures in brackets are the average number of days elapsing between infection and death.

It will be noted that, while the old skin test shows the two cultures tested to be equally virulent, the new test shows a marked difference in their virulence.

SUMMARY.

A method, standardized in all details, is described for measuring the virulence of plague cultures. It employs the Haffkine-Institute-inbred strain of white mouse as the experimental animal and progressive tenth dilutions of 48-hour broth subcultures incubated at 25°C. to 27°C. of the strain of *Past. pestis* under investigation. Three or more dilutions are selected and given to batches of mice in quantities of 0·2 c.c. per animal. The number of organisms in these dilutions are counted by the colony-count method by plating on blood agar. The results are expressed as the smallest number of organisms of a given strain of *Past. pestis* which when given subcutaneously to a batch of mice will kill approximately 100 per cent of the mice infected within 3 to 11 days.

ACKNOWLEDGMENT.

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EXPERIMENTAL STUDIES IN PLAGUE.

Part VI.

A METHOD FOR MAINTAINING THE VIRULENCE OF *PASTEURELLA PESTIS.*

BY

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IN a previous paper (1939*b*) the necessity of a constant test infective dose, for animal experimental work, constant both as regards virulence and the quantity of organisms, was discussed and a method for particularizing the quantity of organisms by enumerating their numbers was described. The problem of achieving constancy of the virulence of the organisms still remained. Haffkine, in the days of the pandemic, relied upon freshly isolated strains from fatal human cases. Even at that time he found that it was not always possible to get fresh strains as often as he desired and had to resort to passage through the guinea-pig. Since he desired to ensure obtaining pure cultures even from contaminated material, he employed the ' cutaneous inoculation ' method for infecting animals for passage. Plague material was rubbed on the shaved and abraded skin of the abdomen of a guinea-pig. Other workers at that time, Gaffkey *et al.* (1899) of the German Plague Commission, Albrecht and Ghon (1900) of the Austrian Plague Commission, and Fraser *et al.* (1900) of the Indian Plague Commission also employed passage through the guinea-pig for maintaining the virulence of their strains. The British Plague Commission (1912) preferred using the house rat (*Rattus rattus*) as the passage animal and also used the ' cutaneous inoculation ' method for infection.

In fact passage through a susceptible animal has been almost the universal method for maintaining the virulence of plague strains, but opinions were not unanimous on the effect of passage on the virulence of the organism. Yersin, Calmette and Borrel (1895) held that though passage through an animal enhanced the virulence of the plague organism for that species it attenuated it for other species. Hankin (1897, quoted by Kolle and Wassermann, 1928) believed that strains passaged through rats became less dangerous for man. Albrecht and Ghon (*loc. cit.*) as well as Kolle and Martini (1902, quoted by Kolle and Wassermann, *loc. cit.*) showed that enhancement of the virulence of a strain obtained by passage through any species was maintained for other species if they were more or less as susceptible as the passage animal. Kolle and Martini (*loc. cit.*), however, noted that passage through the rabbit attenuated the organisms. They held the rabbit body to be unsuitable for the growth of the plague organism. Because of this

observation they were not prepared to rule out the possibility of attenuation of the organism for one species by long continued passage through another species. They however held that there was no such antagonism between man, rat, and mouse. Kolle, Hetsch and Otto (1904) showed that a culture which is avirulent for the rat may still be virulent for the guinea-pig, and that strains that have lost their virulence may regain it by passage through susceptible animals. These views on the whole tended to show that though passage through a highly susceptible animal did not affect the virulence, it was likely to be decreased if the passage animal happened to be resistant.

Until 1933 the practice at the Haffkine Institute was to pass the strains used for preparing plague vaccines and for preparing the test infective dose for experimental purposes through the house rat. The method of ' cutaneous inoculation ' was employed. First the house rat (*Rattus rattus*) caught in the city of Bombay was used, but when it was found that the Bombay city rat had become resistant to plague infection, susceptible house rats were monthly imported from the city of Madras, a town which had not suffered from any severe epidemic of plague during the present pandemic.

When ' cutaneous inoculation ' is resorted to an undetermined quantity of plague organisms is rubbed into a shaved abraded patch of skin of unmeasured area, and it is assumed that if after this massive infective dose the experimental animal dies within a given period of time the strain has maintained its virulence, or if the animal does not die that the strain has lost its virulence. It is very likely that in this method of inoculation hundreds of millions of organisms are used for infecting each animal. How the number of organisms in the infective dose influences the results when testing the virulence of plague organisms is shown in Table I :—

TABLE I.

The effect of number of organisms in an infective dose on the results of virulence measurements.

Number of organisms per house rat given subcutaneously.	Number of rats inoculated.	Number of deaths.	Average number of days elapsing between inoculation and death.
64	24	0	..
64,500	25	1	17·0
132,250,000	25	25	3·4

It will be seen that when the infective dose contained 64,500 organisms or less the strain appeared to be almost entirely avirulent both by its failure to kill all animals and by the prolonged killing time, but when the infective dose contained 132,250,000 organisms the same strain appeared to be very highly virulent both by its power to kill and by the shortness of the killing time. When an accurate method of counting the number of organisms in an infective dose had been worked out (Sokhey, 1939*b*), and a quantitative method for measuring the virulence of plague organisms described (Sokhey, 1939*c*) it was found that the underlying

assumption of the ' cutaneous inoculation ' method was not justified. A massive ' cutaneous inoculation ' very often killed the passage animal (Madras house rat), even when the virulence of the organism was measurably reduced, as has been shown in a previous paper (Sokhey, 1939c).

Burgess (1927) has shown that passage through a vaccinated animal reduced the virulence of plague organisms. Such reduction would also occur if organisms are passaged through a naturally resistant individual. The author (Sokhey, 1937) has already shown that resistance of different individuals of the same strain of *Rattus rattus* differs considerably and therefore if a resistant individual is used for passage, the organism will be reduced in virulence although the ' cutaneous inoculation ' method will not reveal such reduction. There is no method by which one can judge the resistance of an individual animal before using it for passage and this constitutes a great disadvantage of the animal passage method of maintaining virulence.

The observation made by various workers that plague cultures on artificial media retained their virulence undiminished for long periods without any special steps being taken suggested another method for preserving the virulence of plague cultures. Albrecht and Ghon (*loc. cit.*) had noted that most of their cultures on nutrient agar which they had collected in Bombay and which had been exposed to temperatures of 30°C. to 36°C. for about five months while in Bombay and were later stored at 20°C. to 21°C. in Vienna retained their virulence undiminished for 13 to 15 months without animal passage. It is however to be noted that these cultures were at no time continuously exposed to a temperature of 30°C. to 36°C. but that during the stay of these workers in Bombay and during the voyage the daily temperature rose to this height for short periods. Maassen (1903) found that some cultures tended to lose their virulence readily while others did not. The latter, when stored in sealed tubes in an ice-box, were found to be fully virulent two years later. McCoy (1909) and McCoy and Chapin (1912) found that plague cultures on nutrient agar four years after original isolation were still virulent, but when tested three years later had lost their virulence. Wilson (1913, quoted by Francis, 1932) found that two cultures on nutrient agar in sealed tubes which had been stored in an ice-box for over ten years retained their virulence intact. Loffler (quoted by Kolle and Wassermann, *loc. cit.*) recommended the use of horse serum instead of nutrient agar for preserving the virulence of plague cultures. More recently, Francis (*loc. cit.*) reported that a culture on a nutrient-agar slope in a tube sealed with a tight-fitting paraffined cork-stopper and stored at 10°C. for nine years was fully virulent at the end of this period.

Most of these workers tested the virulence of the cultures they were dealing with by using quite massive doses for inoculation, yet the fact seems to emerge that low storage temperatures were possibly helpful for preserving the virulence of plague cultures. For the work to be described it was decided to experiment with rabbit-blood agar as the culture medium and 4° ± 2°C. the temperature of storage. This particular temperature was selected as it was made easily available by the use of a domestic electric refrigerator. The author did not know at the time that even at this low temperature *Past. pestis* continues to grow though very slowly. As soon as this fact became known (Sokhey, 1939a), experiments were started with lower temperatures and the results of these experiments will be reported later. An alternative method of preserving virulence by drying broth cultures at very low temperatures (−20°C. to −30°C.) is also being worked out.

METHOD.

Primary cultures from severe septicæmic human cases are obtained by plating venous blood on agar slopes. After four days' growth at room temperature (26°C. to 32°C.) they are tested for purity, by cultural and biochemical tests, by the method of Gore (Taylor, 1933). After a culture has been found to be pure, its virulence is measured quantitatively; if found highly virulent, i.e., 6 to 12 organisms per Haffkine-Institute-inbred white mouse given subcutaneously kill not less than 80 per cent of the animals used, in an average period of about seven days, a large number of sub-cultures on 5 per cent rabbit-blood agar slopes in test-tubes are made from the primary culture, tubes are sealed on the flame and stored in a refrigerator at 4° ± 2°C. A tube was removed from time to time to measure its virulence. Blood-agar slopes are made by melting nutrient agar, allowing it to cool to about 45°C., and then adding the required amount of defibrinated rabbit blood.

RESULTS.

Since 1932 a number of cultures have been preserved and a few illustrative protocols of the results of periodical measurements of their virulence are given :—

TABLE II.

The virulence of Pasteurella pestis *strain 120/5H* stored at* 4° ± 2°C.

Date of virulence measurements.	Number of organisms given subcutaneously per white mouse.	Results.	Average duration of life after inoculation in days.
29–9–32	27	5/5†	4·4
	270	5/5	4·2
11–4–33	11	5/5	5·6
	112	5/5	4·6
15–5–33	18	5/5	5·4
	188	5/5	3·6
27–11–33	11	4/5	11·5
	..	..	..
23–4–34	12	5/5	6·2
	117	5/5	5·4
25–10–34	8	5/5	12·6
	80	5/5	7·0
19–3–35	8	5/5	11·8
	80	5/5	6·4
4–11–35	8	3/5	9·4
	80	5/5	6·0

* The culture tubes of this particular strain were not sealed on the flame, but were merely plugged with cotton-wool and paraffined. It was found that the cultures dried up when sealed in this manner. For subsequent work sealing of the tubes on the flame was therefore introduced.

† The numerators denote the number of deaths and the denominators the number of mice used.

TABLE III.

The virulence of Pasteurella pestis *strain 34/B stored at* $4° \pm 2°C.$

Date of virulence measurements.	Number of organisms given subcutaneously per white mouse.	Results.	Average duration of life after inoculation in days.
9–5–36 .. {	15	5/5*	5·2
	150	5/5	3·6
21–8–36 .. {	6	5/5	7·0
	67	5/5	4·4
12–2–37 .. {	6	9/10	7·7
	68	5/5	6·0
10–6–37 .. {	13	5/5	5·2
	130	5/5	4·0
20–9–37 .. {	12	5/5	5·8
	120	5/5	4·2
12–1–38 .. {	3	5/5	8·6
	30	4/5	5·6
15–5–38 .. {	8	5/5	5·8
	80	5/5	3·6
16–9–38 .. {	10	5/5	6·8
	106	4/5	4·5
19–12–38 .. {	14	5/5	6·0
	140	5/5	5·2
15–4–39 .. {	9	3/5	10·0
	94	5/5	5·8

* The numerators denote the number of deaths and the denominators the number of mice used.

TABLE IV.

The virulence of Pasteurella pestis *strain 35/B stored at* $4° \pm 2°C$.

Dates of virulence measurements.	Number of organisms given subcutaneously per white mouse.	Results.	Average duration of life after inoculation in days.
18–5–36	13	5/5*	6·4
	137	5/5	4·8
21–8–36	5	5/5	7·2
	59	4/5	5·6
29–1–37	4	4/5	5·8
	46	5/5	5·6
28–5–37	8	5/5	6·8
	85	5/5	3·8
17–9–37	11	5/5	7·0
	112	5/5	4·4
10–1–38	4	5/5	7·2
	40	5/5	6·5
15–5–38	8	5/5	5·6
	80	5/5	5·0
16–9–38	10	5/5	6·4
	106	5/5	3·4
19–12–38	10	4/5	6·0
	100	5/5	5·2
25–4–39	8	4/5	6·8
	79	5/5	4·6

* The numerators denote the number of deaths and the denominators the number of mice used.

TABLE V.

The virulence of Pasteurella pestis *strain 36/H stored at* $4° \pm 2°C.$

Dates of virulence measurements.	Number of organisms given subcutaneously per white mouse.	Results.	Average duration of life after inoculation in days.
18–12–36 ..	11	4/5*	8·3
	112	5/5	7·0
28–5–37 ..	10	5/5	6·2
	107	4/5	6·2
17–9–37 ..	10	5/5	7·2
	102	5/5	5·2
31–1–38 ..	6	4/5	6·8
	62	5/5	6·4
16–5–38 ..	7	5/5	4·4
	70	5/5	4·0
12–9–38 ..	8	4/5	6·0
	86	5/5	6·0
12–12–38 ..	10	5/5	8·0
	100	5/5	4·8
25–4–39 ..	10	5/5	9·2
	106	5/5	8·2

* The numerators denote the number of deaths and the denominators the number of mice used.

TABLE VI.

The virulence of Pasteurella pestis *strain 39/B stored at* $4° \pm 2°C.$

Date of virulence measurements.	Number of organisms given subcutaneously per white mouse.	Results.	Average duration of life after inoculation in days.
31–3–37 ..	11	5/5*	5·8
	116	5/5	4·8
7–7–37 ..	..	..	..
	98	5/5	5·8
29–1–38 ..	11	5/5	6·2
	118	5/5	5·8
12–9–38 ..	10	4/5	5·0
	106	5/5	6·0
16–12–38 ..	12	5/5	9·2
	120	5/5	5·3
1–6–39 ..	9	4/5	5·5
	96	5/5	4·6

* The numerators denote the number of deaths and the denominators the number of mice used.

SUMMARY.

A simple and convenient method for maintaining the virulence of *Past. pestis* cultures is described. It consists in planting the strains to be preserved on 5 per cent rabbit-blood agar slopes in test-tubes, incubating them at room temperature (26°C. to 32°C.) for four days, and at the end of this period sealing the tubes on the flame, and storing them at $4° \pm 2°C.$ Cultures stored in this manner retain their virulence unimpaired for three years and perhaps even longer.

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A SEROLOGICAL STUDY OF SOME STRAINS OF *PASTEURELLA PESTIS.*

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THE main object in undertaking this investigation was to ascertain the serological relationship, if any, between the various strains of *Past. pestis.* The problem of the existence of different strains has been studied by several workers. Wu Lien Teh *et al.* (1936) have summarized the work carried out in the past. Signorelli and Caldarola (1912) and de Smidt (1929) working with strains obtained from different parts of the world came to negative results. Pirie (1927) also admitted that simple agglutination tests failed to reveal the existence of types or groups within the species *pestis* and suggested probable success with agglutinin absorption tests. In this paper we have attempted the serological differentiation by agglutination tests of the various strains grown, especially at room temperature (27°C. to 29°C.), the temperature at which Haffkine broth vaccine is incubated. Some work on strains grown at 37°C. has also been carried out.

AGGLUTINATION TECHNIQUE.

Various agglutination methods have been suggested from time to time in the case of *Past. pestis* but the difficulty of preparing sera of high titre and suitably stable suspensions of the bacilli have always been a hindrance.

1. *Antisera.*—The sera were prepared by inoculating rabbits first subcutaneously and later intravenously with graduated doses of heat-killed (55°C. for 30 minutes) cultures of *Past. pestis* grown at room temperature (27°C. to 29°C.) on ' acid digest agar ' and suspended in normal saline. The first dose consisted of 0·5 c.c. of the saline suspension standardized to Brown's Opacity tube No. 2 subcutaneously, increasing the same to 1 c.c. for the next injection. A similar

dosage was given intravenously and this procedure was followed by substituting live cultures and testing the blood from time to time. Owing to variable agglutinin response of rabbits, no standard course could be followed. Injections were continued until a serum of suitably high titre was produced.

2. *Agglutinable suspension of the bacilli.*—The organism grown at room temperature is salt and serum sensitive, hence useless for preparing a stable suspension. Several ways have been employed to get over this difficulty, e.g., growing at 37°C. (after Dieudonne and Otto, 1927) by suspension of the growth in distilled water (after Kolmer, 1924), by sand filtration (after D'Aunoy, 1923), and by formalinizing the suspension (after Batchelder, 1929). All these methods were tried by Greval and Dalal (1933), but they did not get repeatable results and used the deposit from a broth vaccine grown for eight weeks at room temperature for preparing an agglutinable suspension. This method for preparation of the suspension was used by us in the course of this investigation partly but owing to its preparation taking a long time in case of individual strains and its unusual nature we developed a different technique.

The medium for growth of the cultures is the routine meat-digest agar (pH 6·8) distributed in Roux bottles. These are sown with a thick emulsion of the organisms and incubated at 27°C. or 37°C. for four days according to the nature of agglutinable antigen wanted. The growth is washed with about 20 c.c. to 30 c.c. of distilled water containing 1 per cent of carbolic acid. The washings are poured into a sterilized test-tube containing glass beads and kept at room temperature (27°C. to 29°C.) for six hours with an initial shaking by hand for five minutes and an occasional shaking during the first two hours. The supernatant homogeneous layer is then pipetted off and diluted with an equal volume of normal saline and allowed to stand in the refrigerator overnight allowing coarse particles, if any, to settle down. The supernatant portion is pipetted off and diluted with normal saline containing 0·1 per cent formalin so that the opacity lies between 2 and 3 of Brown's Opacity tubes. Later on this method was modified, the modification consisting in centrifugalizing the stable portion obtained from the suspension kept in the refrigerator and after washing the deposit in saline twice it was re-suspended in normal saline containing 0·25 per cent carbolic acid; formalin 0·05 per cent was added if the suspension was produced in bulk for stock purposes to prevent the growth of fungi. The modification to re-suspend the bacteria was necessary to eliminate any precipitin reaction which may result during the test by using the original liquid in which bacilli were suspended.

In this connection it may not be out of place to mention briefly some of the other methods tried by us but found to give poor or no results. White (1927) found that concentrated alcohol applied at a temperature between 50°C. and 60°C. removed salt sensitiveness of rough Salmonella variants without damaging the essential serological reactions of the bacillary bodies. Similarly, ether and chloroform were found to be effective but acetone was not so. We followed the technique used by White but without success. The use of eight weeks' growth in broth of the organisms by Greval and Dalal (*loc. cit.*) gave us the idea that breaking up of the organism may be the factor concerned. We used the freezing and thawing method for the purpose with no better results. The details of the technique are recorded in the Report of the Haffkine Institute for 1932–35.

We also tried growing the organism on different media and in some cases stable emulsions were obtained but these gave variable results as compared to the suspensions obtained by treatment with 1 per cent carbolic (*vide supra*).

3. *Absorption tests.*—Complete removal of the appropriate agglutinins with minimal amount of the suspensions was aimed at. The serum to be absorbed was diluted ten times. One c.c. of this 1 in 10 dilution was mixed with 1 c.c. of the bacterial suspension containing varying number of organisms (3,000 to 30,000 millions). The mixture was kept in the water-bath at 37°C. for six hours and in the refrigerator overnight. The supernatant clear fluid was removed after centrifugalizing and set up against the agglutinable suspension described above. A serum control containing no suspensions but the serum and saline only was dealt with similarly.

4. *Agglutination tests.*—Macroscopic technique was adopted for the purpose and measurements were made volumetrically. The tests were set up in geometric series starting at a dilution of 1 in 50 and 1 in 20 in the case of 27°C. and 37°C. growths respectively. Readings were made after four hours in the water-bath at 54°C.

TYPE OF AGGLUTINATION.

Two types of agglutination were seen during the course of these experiments, one being specific to room-temperature growths and the other to 37°C. growths of *Past. pestis.* The types of agglutination depend on the agglutinable antigen and not on the type of antisera employed. The main points are summarized below :—

Room-temperature (27°C. to 29°C.) growths.	37°C. growths.
1. Agglutinates slowly.	Agglutinates rapidly.
2. Flakes small and uniform.	Flakes larger and of varying size.
3. Sediment compact.	Sediment voluminous.
4. Sediment not easily dislodged, small flakes seen in a clear fluid on shaking. Clumps take time to re-form and settle down.	Sediment easily dislodged, uniformly distributed on shaking. Clumps re-form after about 10 minutes and settle down in large masses.

STRAINS EMPLOYED.

A brief history and other particulars of the strains employed are given in a tabular form in the *Appendix.*

ROOM-TEMPERATURE GROWTHS.

Preliminary experiments.—As a preliminary, individual antisera were obtained by injecting rabbits as mentioned above with three strains designated as Vimlabai,

34/B, and I. The results of agglutination with the homologous and heterologous agglutinable antigens are summarized in Table I :—

<div align="center">TABLE I.</div>

<div align="center">*Results of agglutination experiments.*</div>

Agglutinable antigen.	ANTISERUM TITRE.			
	Vimlabai.	34/B.	I.	
P ..	400	800	800	
Q ..	400	800*	800	Controls.
R ..	400	800*	800	With normal saline negative in all.
I ..	400	800	800	
34/B ..	400	800	800	With normal rabbit serum negative in all.
35/B ..	400	800	800	
36/H ..	400	800	800	
37/H ..	400*	800	800	
120/5H ..	200*	400*	800	
Egypt ..	400	800	800	
Vimlabai ..	400*	800	800	

* Partial agglutination in the next tube.

The highest dilution which gave definite agglutination is alone recorded for the sake of brevity.

From the study of results set out in Table I it is apparent that the serum prepared from any one of the above strains is capable of agglutinating all other strains practically to titre. It may be inferred that there are no different strains amongst those employed in the above experiments, as judged by the agglutination test.

<div align="center">ABSORPTION EXPERIMENTS.</div>

To corroborate the above findings it was thought necessary to carry out a series of agglutinin absorption tests with antiplague sera prepared from seven strains chosen as representatives from the strains stocked at the Haffkine Institute. Out of the strains used, four were Indian, one Egyptian, one from China, and

one from South Africa. Their particulars are detailed in the *Appendix*. The method for production of antisera and the technique for absorption have already been mentioned above. The results are summarized in Table II :—

<center>TABLE II.</center>

<center>*Results of absorption experiments.*</center>

Antiserum.	Absorbing antigen.	TITRE OF ABSORBED SERUM WITH HOMOLOGOUS AGGLUTINABLE ANTIGEN.	
		Antigen obtained after adding 1 per cent phenol.	Greval's débris suspension antigen.
Egypt ..	Egypt	*Nil.*	*Nil.*
	H	,,	,,
	I	,,	,,
	Vimlabai	,,	,,
	China	400	400
	Saline control	400	400
34/B ..	34/B	*Nil.*	—
	Vimlabai	,,	—
	X	,,	—
	B. pestis (1920)	,,	—
	Java	,,	—
	S. Africa	,,	—
	Saline control	600	—
R	R	*Nil.*	*Nil.*
	H	,,	,,
	I	,,	,,
	Vimlabai	,,	,,
	W	,,	,,
	Egypt	,,	,,
	Saline control	600	400

TABLE II—*contd.*

Antiserum.	Absorbing antigen.	TITRE OF ABSORBED SERUM WITH HOMOLOGOUS AGGLUTINABLE ANTIGEN.	
		Antigen obtained after adding 1 per cent phenol.	Greval's débris suspension antigen.
I	I	*Nil.*	*Nil.*
	W	,,	,,
	China	400	400
	H	*Nil.*	*Nil.*
	Vimlabai	,,	,,
	R	,,	,,
	Egypt	,,	,,
	X	,,	,,
	Saline control	400	300
Vimlabai ..	Vimlabai	*Nil.*	—
	35/B	,,	—
	36/H	,,	—
	W	,,	—
	S. Africa	,,	—
	China	200	—
	I	*Nil.*	—
	Lachmi	,,	—
	Java	,,	—
	X	,,	—
	120/5H	,,	—
	Saline control	400	—
S. Africa ..	S. Africa	*Nil.*	—
	Vimlabai	,,	—
	35/B	,,	—
	36/H	,,	—

TABLE II—*concld.*

Antiserum.	Absorbing antigen.	Titre of absorbed serum with homologous agglutinable antigen.	
		Antigen obtained after adding 1 per cent phenol.	Greval's débris suspension antigen.
S. Africa— *concld.*	W	*Nil.*	—
	I	,,	—
	Lachmi	,,	—
	Java	,,	—
	China	300	—
	X	*Nil.*	—
	R	,,	—
	Saline control	400	—
China B VIII	China	*Nil.*	*Nil.*
	Vimlabai	400	—
	35/B	400	—
	36/H	400	—
	W	400	400
	S. Africa	400	400
	I	400	400
	Egypt	400	400
	Lachmi	400	400
	Java	400	—
	H	400	400
	X	400	—
	R	400	400
	120/5 H (virulent)	400	—
	120/5 H (aviru- lent).	400	—
	Saline control*	600	600

* Controls containing saline in place of suspension and treated similarly.
— means test not put up.

The highest dilution which gave *definite* agglutination is alone recorded for the sake of brevity.

All the strains seem to be identical with each other with the single exception of China B VIII. Its antiserum, when absorbed with heterologous strains, shows a certain loss in its titre exhibiting the existence of a common group agglutinin but it is not possible to remove the agglutinins specific to the China strain itself.

There are some cultural differences between China and other strains as well, e.g., it grows on nutrient agar when put up for the differential growth test, while the other strains do not.

For the differential test a 5-mm. loopful of 48-hour broth culture is added to 4 c.c. of sterile peptone water and one 5-mm. loopful of this dilution is planted on a nutrient-agar slope. This is incubated at 37°C. for 48 hours.

To ascertain any antigenic relation of this strain with *B. pseudotuberculosis rodentium*, the absorption of the China serum was carried out with 27°C. growth of the latter organism. It is apparent from Table III that no such relationship could be established :—

TABLE III.

Antiserum.	Absorbing antigen.	Titre with agglutinable China B VIII antigen (27°C. growth).
China B VIII	*B. pseudotuberculosis rodentium* (27°C. growth).	400
	Saline control.	400

It is possible that China B VIII represents a *Pasteurella* type distinct from *Past. pestis* or a variant of the latter. We are working with five more strains obtained from China and hope to deal with this point fully in a future communication.

EXPERIMENTS WITH STRAINS GROWN AT 37°C.

In the above investigation as stated previously, the cultures for production of antisera and absorption test were grown at room temperature (27°C. to 29°C.) and as Schütze (1932) has pointed out antigenic differences between the cultures grown at 37°C. and 26°C. growths it was thought worth while to carry out some experiments.

The antiserum used was a rabbit serum obtained by immunizing a rabbit with the strain 120/5H (virulent) grown at 37°C. This serum was absorbed as before with cultures grown at room temperature (27°C. to 29°C.) and at 37°C. The strains used were 120/5H virulent and avirulent, 33/B, I, virulent and avirulent, W, X, and China.

The results are summarized in Tables IV and V :—

TABLE IV.

Antiserum.	Absorbing strains (37°C. growth).	Titre with 37 antigen of 120/5H.	Titre with 27 antigen of 120/5H.
120/5H	120/5H (virulent)	*Nil.*	*Nil.*
	120/5H (avirulent)	,,	,,
	33/B	,,	,,
	I (virulent)	,,	,,
	I (avirulent)	,,	,,
	W	,,	,,
	X	,,	. ,,
	China	120†	600
	Saline control*	240	800†

* Control containing saline in place of suspension and treated similarly.

† Partial agglutination in the next tube.

TABLE V.

Antiserum.	Absorbing strains (27°C. growth).	Titre with 37 antigen of 120/5H.	Titre with 27 antigen of 120/5H.
120/5H	120/5H (virulent)	160	*Nil.*
	120/5H (avirulent)	160	,,
	33/B	160	,,
	I	160	,,
	W	160	,,
	X	160	,,
	China	240	600
	Saline control*	240	800†

* Control containing saline in place of suspension and treated similarly.

† Partial agglutination in the next tube.

To corroborate the above observations the experiments recorded in Tables VI and VII were also repeated with a polyvalent antiplague serum (No. 15) prepared at the Haffkine Institute by immunizing horses at first with 37°C. live growth of 120/5H (avirulent) and later with other mixed strains. In this particular case strains used are 12, viz., 120/5H, I, P, R, Vimlabai, X, 34/B, 36/H, 37/B, 38/B, 39/B. Absorption of the serum was carried out with individual strains grown at 37°C. and 27°C. as well as with a mixed suspension of the strains used for production of the serum. The absorbed sera were put up for agglutination with an antigen produced from a mixture of all the strains used for immunization of the horse. The results are summarized in Tables VI and VII :—

TABLE VI.

Antiserum.	Absorbing antigen (37°C. growth).	Titre with poly-valent agglutinable antigen (37°C. growth).	Titre with poly-valent agglutinable antigen (27°C. to 29°C. growth).
	Vimlabai	*Nil.*	*Nil.*
	34/B	,,	,,
	35/B	,,	,,
	W	,,	,,
Polyvalent anti-plague serum (horse No. 15)	S. Africa	,,	,,
	China	120*	800
	I	*Nil.*	*Nil.*
	Lachmi	,,	,,
	Mixed strains	,,	,,
	Saline control	160	1 in 1,200

* Partial agglutination in the next tube.

With an idea of ascertaining whether it was possible to absorb all the agglutinins of a serum produced with strains grown at 37°C. by means of cultures grown at room temperature, the polyvalent serum (No. 15) was absorbed with

room-temperature growths and put up against the agglutinable polyvalent antigen (37°C. and 27°C.). The results are summarized in Table VII :—

TABLE VII.

Antiserum.	Absorbing antigen (room-temperature growth).	Titre with poly-valent agglutinable antigen (37°C. growth).	Titre with poly-valent agglutinable antigen (room temperature 27°C. to 29°C. growth).
	Vimlabai	120	*Nil.*
	34/B	120	,,
	35/B	160	,,
	120/5H	120	,,
	36/H	120	,,
Polyvalent anti-plague serum (horse No. 15).	W	120	,,
	S. Africa	120	,,
	China	120	800
	I	160	*Nil.*
	Lachmi	120	,,
	Mixed strains	120	,,
	Saline control	160	1,200

It would be apparent that room-temperature growth is incapable of absorbing all the agglutinins and that a remnant is always left which can agglutinate a 37°C. growth antigen. From the above tables it is also obvious that no difference in the various strains of *Past. pestis* (grown at 37°C.) exists except in the case of China B VIII. Some remarks regarding the antigenic structure of the plague bacillus, however, seem to be relevant. The interpretation of the above experiments becomes easier if we adopt the conception of Schütze (*loc. cit.*) that the *plague bacillus* possesses two antigens—the somatic and the envelope, the former being heat-stable while the latter is heat-labile (destroyed when heated at 100°C. for one hour). It is apparent from the tables that the 37°C. growth is capable of absorbing all the antibodies of an immune serum produced with 37°C. growth, the absorbed serum showing no agglutination with the agglutinable antigens (37°C. or 27°C.). On the other hand room-temperature growth cannot remove entirely the antibodies from the same serum leaving always a remnant which agglutinates with the 37°C. agglutinable antigen. Evidently the 27°C. growth contains mostly somatic antigen though some envelope antigen must be present as the titre of the serum (37°C.) is reduced to some extent when it is absorbed with a suspension of 27°C. growth.

The following experiment shows the effect of heat on the 37°C. growth :—

Antiserum.	Absorbing strains (37°C. growth).	Titre with 37 antigen of 120/5H.	Titre with 27 antigen of 120/5H.
120/5H (37°C. serum).	120/5H unheated	*Nil.*	*Nil.*
	120/5H heated for 1 hour at 100°C.	240	,,
	Saline control	240	800

This also corroborates the existence of two antigens in the 37°C. growth (heat-labile and heat-stable), the heated growth acting somewhat similar to the room-temperature culture. The antigenic differences can also be inferred from the type of agglutination specific to incubator (37°C.) and the room-temperature (27°C. to 29°C.) growth of *Past. pestis.* The rapidity of agglutination, the size of flakes, and the nature of sedimentation are characteristic of the particular type of growth. It is also apparent from these tables that by means of the above agglutination and absorption tests it is not possible to differentiate between a virulent and an avirulent strain ; they seem to be serologically identical.

SUMMARY AND CONCLUSIONS.

1.   An attempt has been made to study the serological relationship between some representative strains of *Past. pestis.*

2.   The agglutination and agglutinin absorption test have been employed during the investigation and a new technique for preparation of the agglutinable antigen has been described.

3.   Types of agglutination specific to room-temperature (27°C. to 29°C.) and the incubator (37°C.) growth have been recorded.

4.   Neither in the case of room-temperature (27°C. to 29°C.) nor in the case of the incubator (37°C.) growth, could any serological differences be detected between the various strains of *Past. pestis*, the only exception being a strain obtained from China (China B VIII). This strain may be a *Pasteurella* type distinct from *Past. pestis*, further investigation on the point is being continued.

5.   The existence of the types of antigens in *Past. pestis* when grown at 37°C. or room temperature have been discussed.   The incubator (37°C.) growth is capable of absorbing all agglutinins from the sera obtained by immunizing animals with incubator or room-temperature growths, whereas the room-temperature growth is only capable of removing the antibodies from a serum produced by immunizing animals with room-temperature growth.   In the case of 37°C. growth antisera a residual portion is always left when the same is absorbed with 27°C. growth suspension.   The incubator (37°C.) growth when heated at 100°C. for one hour behaves more or less like a room-temperature growth.   Antigenic differences are

also shown by the characteristic type of agglutination of the 37°C. and the room-temperature cultures.

6. Using the technique detailed in the paper it was not possible to detect any serological differences between the avirulent and virulent types of the strains.

ACKNOWLEDGMENT.

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APPENDIX.

History and other data of the strains employed for the experiments.

Name of strain.	Serial number.	Source.	Date of isolation.	Whether satisfying routine tests.	Number of the sub-culture used.	Virulence test by Sokhey and Maurice (1935).	
						Number of organisms used.	Average duration of life.
..	36/H	Hyderabad (blood culture).	3–12–36	Satisfied the routine tests of plague.	1st	11 5/5 110 5/5	9¼ days.
1 ..	18/B	United Provinces W. P. (blood culture).	31–3–34	do.	2nd	7 5/5	8 „
Vimlabai ..	33/B	Bombay Isolation Hospital (blood culture).	23–1–36	do.	1st	8 5/5 80 5/5	7 „ 7 „
Abdul Vahab ..	34/B	Bellary, Madras Presidency (blood culture).	21–1–36	do.	1st	15 5/5 150 5/5	6 „ 6 „
Mohanbee ..	35/B	do.	22–1–36	do.	1st	14 5/5 137 5/5	7·4 „ 7·8 „
	120/5H	Hyderabad (blood culture).	29–9–32	do.	3rd	6 3/5 65 4/5	12 „
X ..	23/H	Hoshiarpur, Punjab (blood culture).	28–12–34	do.	2nd	9 5/5 90 5/5	9 „ 8 „
R ..	21/H	Hyderabad-Deccan (blood culture).	26–1–35	do.	2nd	7 5/5 70 5/5	7·2 „

	No.	History / source	Date isolated	Biochemical reactions	3rd	Virulence (0·5 c.c. of 48-hour broth culture)
W	19/H	do.	7-1-35	do.	do.	4 4/5 || 8 " / 40 3/5
S. Africa	30/A	Kimberly, S. Africa (spleen culture).	Isolated in 1925. Received from Dr. Harvey Pirie, Lister Institute, London.	do.	Not known	0·5 c.c. of 48-hour broth culture did not kill a rat.
Java	32/J	Java (blood culture).	Isolated in 1925 by Dr. L. Otten, Pasteur Institute, Bandoeng.	do.	Not known	do.
Egypt	31/E	Beba, Cairo, Egypt (bubo culture).	Isolated by Col. H. Marrian Perry in 1929.	do.	Not known	do.
H	Nil	Borsad, Gujrat (blood culture).	7-3-35	do.	..	..
Past. pestis 1920	Nil	Not known	1920	do.	do.	Not known.
China	BVIII	Peiping, China. National Epidemic Prevention Bureau, isolated at Herbin in 1919.	Received from Dr. S. N. Tao in April 1935.	Satisfies the carbohydrate and the protein reactions. When put up for the differential test it, however, shows profuse growth on standard agar, while others do not show any growth.	..	0·5 c.c. of 48-hour broth culture did not kill a rat.
Lachmi	..	Hyderabad-Deccan	Received from the Special Plague Officer, Hyderabad-Deccan, in January 1939.	Satisfies all the routine tests of plague.	..	Not done.
120/5H	120/5H	..	..	..	..	..
Avirulent	Avirulent	Made avirulent by repeated sub-culturing at 37°C.			..	..

THE RELATIVE VALUE OF SOME PROPRIETARY CYANIDE PREPARATIONS FOR THE EXTERMINATION OF RATS AND FLEAS AS A PLAGUE-PREVENTIVE MEASURE.

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RAT AND FLEA extermination forms the most important plague-preventive measure, but its effective execution especially in human dwellings has always presented serious difficulties. Dealing with the problem by rat proofing of dwellings and building suitable godowns for the storage of grain is not economically possible in India, more so as the disease has become almost entirely a rural problem. Poison baiting and trapping, the measures usually resorted to, are not very successful. The rats soon learn to avoid these dangers. Similarly, the usual methods of dealing with fleas in dwellings by sprinkling emulsion of kerosene oil or pesterine are even less successful. Fumigation with hydrocyanic acid gas has always proved to be the most effective method of dealing with both rats and fleas, but so long as the only way was, to have recourse to the gas as such the process was dangerous and did not lend itself to wide use. With the availability of new proprietary cyanide compounds, which are very stable and give out HCN only when blown into the air, cyanide fumigation of dwellings became a practical proposition. In this form HCN fumigation is relatively safe and easy to apply. These preparations make hydrocyanic acid gas the fumigant of choice, and they afford a quick and certain method of destroying both rats and fleas at the same time. Some of these preparations have been in use for some time now and their use is extending. It was, therefore, considered worth while to determine by experiment which of them was best suited for antiplague work under Indian conditions.

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The cyanide preparations tested.*

1. Cyanogas A Dust (prepared by the American Cyanamid Co., New York) is a very finely divided greyish blue powder about 80 per cent of which passes a 200 mesh screen, and is said to contain not less than 40 per cent and not more than 50 per cent of pure calcium cyanide. One sample used for some of the experiments detailed in this paper on analysis showed a $Ca(CN)_2$ content of 42·30 per cent and an HCN content of 23·31 per cent.

It is sold on the market in 5 lb. and 20 lb. tins, and 100 lb. barrels. The makers also provide a foot-pump for the application of the Dust—Cyanogas Foot-Pump Duster. It is a large-bore pump to the nozzle of which is securely fastened a glass-jar which holds the Dust. It is so constructed that it can be held in place with a foot and both hands are free for the operation of the pump. On the downward stroke of the piston, air is suddenly blown into the glass-jar and thus a cloud of dust is created and blown out of the jar through an outlet to which is attached a rubber hose which can be introduced into a burrow or a room as desired.

The makers (and also an Indian firm †) are now providing a modified pattern of their original foot-pump duster. This pattern is provided with a ' cut-out' device. With this ' cut-out' device the pump can be used to blow air independently of the Dust if desired. This ' cut-out' device is of particular value when the Dust is applied to rat burrows, as we shall show below.

2. Calcid Briquettes (prepared by Deutsche Gesellschaft für Shädlingsbekämpfung, Frankfurt A. M.) are small tablets, each weighing about 20 g., of pressed calcium cyanide. They are said to contain about 88·5 per cent of pure calcium cyanide. One of the samples used in the tests to be described below · showed on analysis to have a $Ca(CN)_2$ content of 84·94 per cent and an HCN content of 46·80 per cent. The Calcid Briquettes are sold usually in 4 kg. sealed tins.

Three special blowers for using the preparation are provided by the makers. All these machines are combined grinders and blowers. The turning of the handle of the machine grinds the Briquettes into fine powder and the powder so resulting is blown out by the blast produced by the fan which is fixed on the same axis. The smallest of the blowers is the small Calcid Baby Blower which has a fan of 5½ inches diameter. The next size has a fan with blades 7½ inches in diameter. The largest is Type 6 Degesch Machine which has a fan diameter of 10 inches.

3. Cymag (prepared by the Imperial Chemical Industries. Ltd., London) is a finely ground powder over 60 per cent of which passes 200 mesh screen. Its composition is not disclosed, but it is said to contain 20 per cent HCN. One of the samples used in this work was found to have an HCN content of 19·78 per cent. It is sold usually in 7 lb. tins.

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* The following local representatives of the makers presented the Haffkine Institute with samples of their products and requested their suitability to be tested :—
1. Messrs. Shaw Wallace & Co., Bombay—Cyanogas A Dust.
2. Messrs. Havero Trading Co., Bombay—Calcid.
3. Messrs. The Imperial Chemicals, Bombay—Cymag.

† Messrs. Bali & Co., 168 Chandni Chowk, Delhi.

The makers provide a small portable blower* — Early Bird Blower. The blower available in Bombay was not found satisfactory, and in experiments reported below the new pattern Cyanogas Foot-Pump Duster was used for applying Cymag.

MEASUREMENT OF THE YIELD AND RATE OF EVOLUTION OF THE GAS FROM THE PREPARATIONS UNDER TEST.

The description of the three preparations given above tells us only about their total content of HCN. But what is important from our point of view is to know the actual amount of HCN that these preparations yield when applied. It is equally important to know the rate at which the gas is evolved from each of the three preparations. The importance of the rate of evolution of gas will become clear, when we come to describe actual experiments in connection with the disinfestation of huts.

The evolution of HCN gas from calcium cyanide is a simple process. When finally divided calcium cyanide powder comes in contact with atmospheric air, which normally contains a larger amount of water vapour, it gives up HCN and the calcium drops down as inert slaked lime.

To measure the amount and the rate of evolution of HCN from the preparations under test a specially built air-tight chamber was used. The sides and the roof of the chamber were made of plates of glass held in place in a wooden frame. One side was provided with a door also made of glass-plates. The floor was made of wood boards dovetailed into each other and pasted over with thick cartridge paper. All the joints and door were made air-tight with strips of felt. To prevent chance leaks the door was also pasted over with strips of paper. The dimensions of the chamber were, height 6 feet 6 inches, length 7 feet 6 inches, and width 4 feet. One side wall of the chamber was provided with two wooden recesses for another experiment and deducting the space occupied by these recesses the capacity of the chamber was 200 cubic feet.

The chamber was provided with glass-tubes let in from one side to permit of samples of enclosed air being aspirated. Similarly, a large-bore inlet tube was provided to permit the applications of the fumigants under test to the interior of the chamber. This inlet tube opened 2 inches above the centre of the floor.

For each test a given amount of the fumigant was blown into the chamber through the inlet tube. This process took about 15 seconds. Thereafter at stated intervals three samples of air were simultaneously withdrawn from three tubes, one 76 inches, the second 50 inches and the third 6 inches above the level of the floor. Each sample measured 5 litres. It took 90 seconds to draw each sample, therefore the aspiration of the sample was begun 45 seconds earlier and completed 45 seconds after the time intervals given in the table. The aspirated air was passed through two wash bottles containing 5 per cent solution of caustic soda and the resultant solution was titrated against silver nitrate using potassium iodide as the indicator. The average HCN content of the three samples simultaneously drawn each time is given in the protocol.

The results of the determinations are given in Tables I and II :—

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* Only one blower was tested which proved to be mechanically defective, in that there was a back draft which blew some of the dust backwards. The makers, however, assure us that Early Bird Blowers are perfectly sound and give a very powerful blast.

Table I.

The amount and rate of evolution of HCN gas from Cyanogas A Dust, Calcid, and Cymag.

Fumigant.	Quantity introduced into chamber.	Theoretical yield of HCN, expressed as parts per million.	Mean temperature at the time of experiment.	Relative humidity in the chamber at the time of experiment.	HCN ACTUALLY OBTAINED, EXPRESSED AS PARTS PER MILLION AND AS PERCENTAGES OF THE THEORETICAL CONTENT.					
					INTERVALS AFTER THE INTRODUCTION OF THE FUMIGANT INTO THE CHAMBER.					
					5 minutes.	15 minutes.	30 minutes.	45 minutes.	60 minutes.	120 minutes.
Cyanogas A Dust	43 g.	1,660	20°C.	76%	1,260 (76%)	1,390 (84%)	1,620 (98%)	.. ..	1,520 (92%)	1,460 (88%)
Calcid ..	40 g.	2,500	30°C.	75%	2,370 (94%)	2,450 (98%)	2,440 (98%)	.. ..	2,340 (94%)	2,275 (91%)
Cymag ..	44 g.	1,320	20°C.	75%	210 (18%)	435 (33%)	487 (37%)	520 (39%)	533 (40%)	459 (35%)

TABLE II.

Further experiments carried out to measure the amount and rate of evolution of HCN gas from Cymag.

Experiment number.	Quantity of Cymag introduced into chamber.	Theoretical yield of HCN expressed as parts per million.	Mean temperature at the time of experiment.	Relative humidity in the chamber during the experiment.	HCN ACTUALLY OBTAINED, EXPRESSED AS PARTS PER MILLION AND AS PERCENTAGES OF THEORETICAL CONTENT. INTERVALS AFTER THE INTRODUCTION OF THE FUMIGANT INTO THE CHAMBER.								
					5 minutes.	15 minutes.	30 minutes.	45 minutes.	60 minutes.	90 minutes.	120 minutes.	180 minutes.	240 minutes.
1	50·2 g.	1,420	30°C.	64%	321 (23%)	393 (28%)	482 (34%)	553 (39%)	590 (42%)	699 (49%)	650 (46%)	545 (38%)	512 (36%)
2	43·8 g.	1,210	30°C.	68%	264 (22%)	411 (34%)	500 (41%)	530 (44%)	553 (45%)	585 (48%)	542 (45%)	520 (43%)	421 (35%)

It will be noted that both Cyanogas A Dust and Calcid on coming in contact with air give up their total content of HCN. In a closed space the maximum yield in the case of Calcid is reached in 15 minutes, while in case of Cyanogas A Dust it is reached in 30 minutes, showing that evolution of the gas is twice as fast from Calcid as from Cyanogas A Dust. Cymag yields only about half of its total content of HCN and the rate of evolution is very slow. It takes 90 minutes to reach even this low concentration *.

<div align="center">

EXPERIMENTS TO DETERMINE THE RELATIVE EFFECTIVENESS OF THE FUMIGANTS UNDER TEST.

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In actual antiplague operations in the field one is called upon to disinfest rat burrows which may be blind or open and to disinfect dwellings and other buildings as a whole. The dwellings may vary from *pucca* structures with well-fitting doors and windows capable of being easily rendered air-tight to flimsy huts which cannot be made even partially air-tight. The experiments detailed below were designed to test the value of these proprietary cyanide preparations under all the usual conditions met with in the field.

1. EXPERIMENTS WITH BURROWS.

Description of the burrow.—For these experiments an artificial burrow dug in the ground was employed. It consisted of a channel 4 inches wide. 3 inches deep, and 240 inches long, consisting of 4 equal portions (each 60 inches in length), arranged in the form of a W, the angle of inclination between each portion being 45°C. Pieces of expanded metal of $\frac{1}{2}$-inch mesh, were fixed across the channel near the angles. at ' b ', ' c ', and ' d ' as shown in the Diagram, to divide it into four sections, ' A ', ' B ', ' C ', and ' D '. The whole channel was roofed in with plates of glass held in place with mud. The opposing edges of glass-plates were made air-tight with Plasticine. A small window for introducing rats and fleas into the compartment was cut in the centre glass-plate of the roof of each section. These windows were closed with small plates of glass fixed in place with Plasticine. The rat burrow made in this fashion proved to be reasonably air-tight. Its capacity was 1·66 cubic feet.

Method of testing.—For each test two adult house rats and a muslin bag containing five fleas were introduced through the windows in the roof into each section of the burrow except section ' A '. No rats and fleas were introduced into section ' A ' as it had the inlet for cyanide preparation and unnecessarily large amount of the powder deposited itself in this section. Thus 6 rats and 15 fleas were used for each test. End ' e ' of the burrow was closed with a piece of expanded metal, $\frac{1}{2}$-inch mesh, when it was desired to use the burrow as an open burrow, but this end

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* The Imperial Chemical Industries, Ltd., London office repeated Cymag tests under the conditions used at the Haffkine Institute, and results similar to those found in Bombay were recorded. The makers remark, ' In the Haffkine (Institute) method the air is *static* and it appears that with Cymag a state of equilibrium occurs when the atmosphere contains 30 to 40 per cent of available HCN (depending on the amount of Cymag present)'.

was closed with pieces of stone and mud, when the burrow was used as a blind burrow. The nozzle of the rubber hose of the appropriate blower was inserted into the opening at ' *a* ' and the rest of the opening carefully blocked with pieces of stone and mud. The burrow was carefully examined to see that glass-plates closing the window were fixed in place and the whole burrow was reasonably air-tight.

DIAGRAM OF THE ARTIFICIAL BURROW.
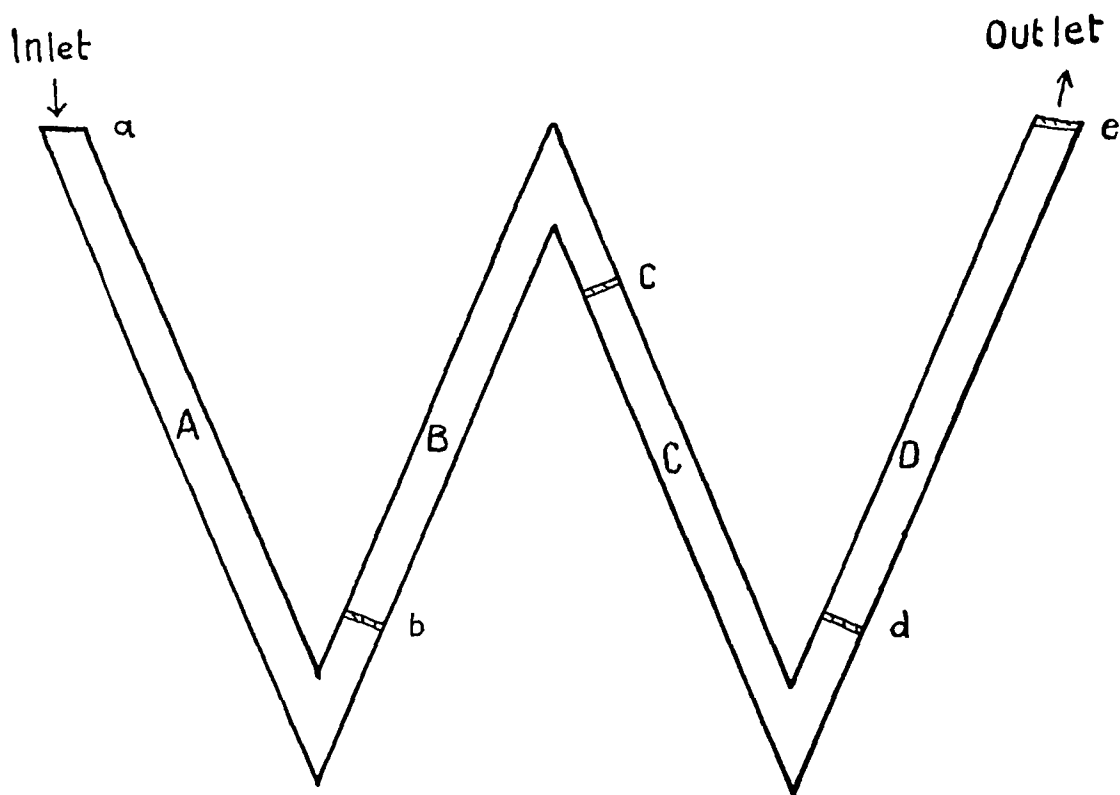
The fumigant in each test was blown into the burrow from the end ' *a* '. When the burrow was used as an open burrow, the opening at the end ' *e* ' was watched and was blocked quickly with mud on the first signs of the escape of the powder from that end. But the blowing in of the powder was continued until a pre-determined amount of it had been blown in. Thereafter if the blower permitted it, as in the case of Calcid Blowers or the new pattern Cyanogas Foot-Pump Duster with the ' cut-out ' device, a certain amount of air was blown in to carry the powder to the farthest portion of the burrow,

In the case of Cyanogas A Dust and Cymag the glass container of the foot-pump was filled to three-quarters of its capacity with the powder, weighed, and then screwed in place. A pre-determined number of strokes of the piston, calculated to blow out a given quantity of the powder, were given. But the actual amount of the powder blown in was determined by weighing the glass container at the end of the operation and by subtracting this weight from the original weight.

In the case of Calcid Briquettes the desired amount was weighed and placed in the blower and the blower worked until the preparation was completely blown in and thereafter a few turns of the handle were given to blow in some air to carry the powder to the farthest part of the burrow.

The blowing in of the fumigants into the burrow took only a few seconds. The time was noted when the blowing in of the fumigant was started and the fumigant was allowed to act for two hours. During this period watch was kept to note the time which the rats took to die. At the end of the two-hour period the roof plates of the burrow were removed and the rats and the muslin bags containing fleas were removed. The flea bags were emptied into a clean dry bell-jar, and the rats were transferred to a cage. The number of rats and fleas that had died was noted.

A. *Experiments with the open burrow.*

For this purpose the artificial burrow described above was used with the outlet ' e ' open to the outside air but blocked with a piece of $\frac{1}{2}$-inch mesh expanded metal to prevent the rats from escaping from section ' D ' of the burrow.

Cyanogas A Dust was applied both with (i) the original pattern Cyanogas Foot-Pump Duster *without* the ' cut-out ' device and (ii) the new pattern Cynogas Duster *with* the ' cut-out ' device. Cymag was applied with the new pattern Cyanogas Duster only. Calcid was blown in with the Calcid Baby Blower with $7\frac{1}{2}$ inches blades.

When the blowing in of the fumigant was commenced a watch was kept on the outlet ' d ' and at the first sign of escape of the powder from that end, it was quickly closed with mud. Blowing in of the fumigant was continued until a pre-determined amount was blown in and thereafter a few more turns of the handle of the Calcid Blower or a few more strokes of the Cyanogas pump were given, if the pump was fitted with a ' cut-out ' device, to blow in a little air to carry the powder to the farthest part of the burrow.

The fumigant was allowed to act for two hours and then the roof glass-plates were removed to take out the rats and fleas. During this period of two hours a watch was kept to note the time of death of the individual rats. Results obtained are given in Table III.

It will be noted that 2·5 g. of Calcid were enough to kill all the animals and the time taken to kill was four minutes, while 9·5 g. of Cyanogas A Dust were required when used with the new pattern pump and the time taken to kill was 4 to 18 minutes. When Cyanogas was applied with the old pattern pump without the ' cut-out ' device as much as 66·5 g. were needed to achieve the same results,

TABLE III.

Experiments with the open rat burrow.

Number of experiment.	Mean temperature, °C.	Relative humidity, per cent.	Number of strokes of piston to apply fumigant.	Number of strokes of piston to blow in air.	Quantity of fumigant applied in grammes.	Time in minutes when last rat ceased breathing.	Results.* Rats.	Fleas.
1. (i) Cyanogas A Dust—applied with the original model Pump without the 'cut-out' device.								
SERIES XI.								
8	29·5	68·0	25	..	92·5	4	6/6	15/15
9	29·5	67·5	20	..	68·5	4	6/6	15/15
4	28·9	66·0	25	..	66·5	9	6/6	15/15
5	29·1	66·5	20	..	56·0	..	5/6	14/15
6	28·2	64·0	15	..	38·0	..	4/6	11/15
7	28·2	64·0	10	..	33·2	..	2/6	7/15
1. (ii) Cyanogas A Dust—applied with the new model Cyanogas Foot-Pump with 'cut-out' device.								
SERIES X.								
10	28·7	61·5	7	20	21·5	5	6/6	14/14
9	28·5	73·5	7	20	17·5	2·5	6/6	15/15
12	29·3	68·5	4	20	9·7	Not noted	6/6	15/16
8	28·9	66·0	7	20	9·5	18	6/6	15/15
5	28·9	63·0	4	20	8·0	..	4/6	12/15
7	28·4	61·0	6	20	6·5	..	5/5	14/15
14	29·5	68·0	3	20	5·9	8	6/6	14/15
13	29·3	68·5	3	20	5·8	11·5	6/6	13/14
11	29·5	70·0	4	20	5·2	4	6/6	12/12
6	28·4	61·0	5	20	5·0	..	5/6	13/15
4	28·9	63·0	3	20	5·0	..	3/6	12/15
3	..	60·5	2	21	2·5	..	2/6	12/15
2	..	60·5	1	20	1·5	..	2/6	9/15

* The numerators indicate the number of deaths and the denominators the number of animals used.

TABLE III—*concld.*

Number of experiment.	Mean temperature, °C.	Relative humidity, per cent.	Number of strokes of piston to apply fumigant.	Number of strokes of piston to blow in air.	Quantity of fumigant applied in grammes.	Time in minutes when last rat ceased breathing.	Results.* Rats.	Results.* Fleas.
2. *Cymag—applied with the new model Cyanogas Foot-Pump with 'cut-out' device.*								
SERIES XIII-A.								
1	29·2	66·5	15	20	34·0	..	2/6	9/15
2	29·5	70·0	30	20	55·8	..	3/6	10/14
4	29·1	68·0	15	20	60·8	..	2/6	7/15
3	29·5	65·5	50	20	90·0	..	5/6	15/15
5	27·5	75·0	142	20	120·0	5th rat died in 8 minutes.	5/6	15/15
3. *Calcid—applied with 7½ inches Calcid Baby Blower.*								
SERIES XII.								
4	28·0	72·5	..	..	3	1·5	6/6	15/15
5	28·7	67·5	..	..	2·5	2·5	6/6	15/15
3	28·0	72·5	..	..	2·5	3·25	6/6	15/15
2	27·2	50·5	..	..	2	4	6/6	14/15
1	27·2	50·5	..	..	1	..	5/6	13/15

* The numerators indicate the number of deaths and the denominators the number of animals used.

In the case of Cymag, even though applied with the new pattern Cyanogas pump, as much as 120 g. failed to kill all the animals. Because of these unsatisfactory results further experiments were not conducted with this product.

Calcid is therefore found to be the most effective of the three preparations under the conditions of the experiment both as regards the smallness of the quantity needed and the shortness of the time taken to kill all the animals. The HCN content of Cyanogas is about half that of Calcid, thus about 4 g. of it should have been enough, but the results with that amount were somewhat uncertain.

B. *Experiments with a blind burrow.*

The same burrow as described above was used except that the outlet ' e ' was closed with stones and mud before the blowing in of the fumigant was started. Other observations were made in exactly the same way as with the open burrow.

The results obtained are given in Table IV.

The quantities of Calcid and Cyanogas A Dust needed to kill all the animals in a blind burrow were Calcid 1·25 g., Cyanogas A Dust 122·0 g. (when applied with the old pattern duster) and 11·8 g. (when applied with the new pattern duster with the ' cut-out ' device). Here too Calcid proves to be more effective than Cyanogas A Dust. If the action had been directly proportional to their content of HCN and if 1·25 g. of Calcid proved enough, 2·50 g. of Cyanogas should have produced the same results, but actually over 7 g. of Cyanogas A Dust were required.

These experiments also make it abundantly clear that the original pattern Cyanogas Foot-Pump Duster *without* the ' cut-out ' is utterly useless for burrow fumigation. The Cyanogas Foot-Pump Duster does not cause a powerful draught and where it is not provided with a cut-out arrangement by which air can be blown in independently of the Dust, most of the Dust is deposited in the first arm, ' A ', of the burrow and thus most of it remains ineffective.

II. EXPERIMENTAL FUMIGATION OF DWELLINGS AS A WHOLE.

Three types of dwellings were selected: (*a*) those that can be made reasonably air-tight, (*b*) those that can be made only partially air-tight, and (*c*) those that cannot be rendered even partially air-tight.

(*a*) *Dwellings capable of being rendered reasonably air-tight.*—Well-built masonry and cement structures with flat impervious roofs and *pucca* floors with well-fitting windows and doors would come under this category. Such dwellings are rare in rural India. But experiments were conducted in one such building in the interest of completeness of our study.

For this experiment one of the rooms of this Institute with a capacity of 11,794 cu. ft. was selected. Twelve rats, each in a separate cage, were placed at different levels in the room. Twelve muslin bags containing two fleas each were similarly distributed. Doors and windows were closed and the hose of the blower was introduced into the room through a circular hole made in one of the glass-panes of the door and fixed in place with Plasticine.

TABLE IV.

Experiments with blind rat burrow.

Number of experiment.	Mean temperature, °C.	Relative humidity, per cent.	Number of strokes of piston to apply fumigant.	Number of strokes of piston to blow in air.	Quantity of fumigant applied in grammes.	Time in minutes when last rat ceased breathing.	Results.* Rats.	Results.* Fleas.
2.		(*i*) *Cyanogas A Dust—applied with the original pattern Cyanogas Foot-Pump Duster without the 'cut-out' device.*						
Series XVI.								
3	32·5	53·5	50	..	140·0	4th rat died in 3 minutes	4/6	15/15
6	29·0	73·0	55	..	137·0	3·0	6/6	15/15
5	28·5	80·0	50	..	135·0	6·0	6/6	15/15
4	29·0	64·0	60	..	122·0	3·5	6/6	15/15
2	28·5	57·0	35	..	84·0	..	4/6	10/15
1	30·5	53·5	25	..	65·0	..	4/6	10/15
2.		(*ii*) *Cyanogas A Dust—applied with the new pattern Cyanogas Foot-Pump Duster with 'cut-out' device.*						
Series XV.								
1	..	..	3	78	14·5	4·0	6/6	15/15
2	15·6	72·0	6	89	17·0	2·5	6/6	15/15
4	24·8	63·0	1	103	11·8	3·2	6/6	15/15
3	25·9	66·0	1	99	7·7	..	5/6	15/15
2.		(*iii*) *Calcid—applied with Calcid Baby Blower.*						
Series XIV.								
5	26·1	..	..	..	2·00	2·5	6/6	15/15
6	26·1	56	..	..	1·50	3·5	6/6	15/15
3	26·1	53	..	..	1·50	2·0	6/6	15/15
16	30·7	55	..	..	1·25	Not noted	6/6	15/15
15	29·6	65	..	..	1·25	3·2	6/6	15/15
7	26·2	66	..	..	1·0	..	5/6	13/15
4	27·2	53	..	..	1·0	..	4/6	10/15

* The numerators indicate the number of deaths and the denominators the number of animals used.

Calcid was applied with the largest Calcid Blower Type 6 Degesch Machine, and Cyanogas A Dust was blown in with the original type of Cyanogas Foot-Pump Duster without 'cut-out' device. After the introduction of the fumigant under test, the room was left closed for six hours. Results are given in Table V:—

TABLE V.

Experiments with an air-tight room.

Number of experiment.	Mean temperature, °C.	Average relative humidity, per cent.	Quantity of fumigant applied in g. per 1,000 cu. ft.	Period of exposure, hours.	RESULTS.*	
					Rats.	Fleas.

(i) Calcid.

SERIES IX-C.

1	32·5	69 0	63	6	12/12	24/24
4	30·5	71·0	30	6	12/12	24/24
2	32·0	66·0	25	6	12/12	24/24
3	32·0	67·0	15	6	7/12	24/24

(ii) Cyanogas A Dust.

SERIES IX-D.

1	31·1	65·0	200	6	12/12	23/23
3	30·2	72·0	90	6	12/12	23/23
2	28·4	74·5	60	6	12/12	23/23
4	30·3	74·0	50	6	12/12	22/22
5	28·0	86·0	40	6	8/12	22/22

* The numerators denote the number of deaths and the denominators the number of animals used.

Of Calcid 25 g. and of Cyanogas A Dust 50 g. were needed to kill all the rats and fleas placed in the room. These quantities are directly proportional to the HCN content of these preparations.

(b) Dwellings capable of being made only partially air-tight.—The dwellings of the better classes in relatively prosperous villages are of this type. They consist of masonry structures with tiled roofs which are not impervious. The windows and doors are usually ill-fitting and there are numerous chinks. For this experiment the Institute motor garage was used. This is a room with brick-walls, with a tiled roof and an ill-fitting large door. The capacity of the room is 1,800 cu. ft.

Cotton waste was used to stuff the chinks, and sheets of paper were used to stop up the interval between the eaves and walls and strips of paper were applied to the door. The product under test was blown in and the room left closed for six hours. The results are given in Table VI :—

TABLE VI.

Experiments with partially air-tight room.

Number of experiment.	Mean temperature, °C.	Average relative humidity, per cent.	Quantity of the product applied in g. per 1,000 cu. ft.	Period of exposure, hours.	Results.*	
					Rats.	Fleas.

(i) *Calcid.*

SERIES III.

6	25·5	86·0	67	6	6/6	14/14
9	25·5	93·0	56	6	6/6	17/17
7	27·7	89·0	44	6	6/6	18/18
8	..	..	33	6	2/6	16/18

(ii) *Cyanogas A Dust.*

SERIES III-A.

1	30·0	78·0	111	6	4/6	18/18
2	30·5	78·0	167	6	5/6	17/17
3	31·5	72·0	222	6	5/6	18/18
4	30·2	74·0	278	6	6/6	18/18

* The numerators denote the number of deaths and the denominators the number of animals used.

Of Calcid 44 g. per 1,000 cu. ft. were enough to kill all the animals, but of Cyanogas A Dust as much as 167 g. were needed. According to the HCN content of Cyanogas A Dust (which is about half of Calcid) 88 g. of it should have achieved the same result as 44 g. of Calcid. The discrepancy, as we shall show in the next experiment, is due to the rate at which HCN gas is evolved from the two products.

(c) *Experiments with dwellings which cannot be made even very partially airtight.*—Most of the dwellings in the villages are of this type. They are usually mud-walled huts with thatched roofs, with or without shutters for doors. Even when shutters are used they are often no more than a few pieces of bamboo tied together. Such huts usually have no windows, chinks in the walls, and the largish interval

between the eaves and the top of the walls serves the purpose of windows. Fumigation of this type of dwelling is in our opinion the crucial test of the value of these fumigants.

For this experiment we selected a dilapidated mud-walled hut with a thatched roof. The hut had two doors without shutters. There was an interval of more than one foot between the eaves and the walls. The hut was divided into two cubicles by a low mud partition and had a capacity of 2,500 cu. ft. We threw two large tarpaulins over the roof, but these tarpaulins were not large enough to completely cover the walls. An occasional hole here and there was stuffed with cotton waste. The rats in individual cages were placed in different parts of the huts at different levels and fleas in muslin bags were similarly distributed. The doors were then closed with pieces of tarpaulin.

Calcid was applied with the large Calcid Blower Type 6 Degesch Machine. The outlet end of the hose from the blower was suspended from the roof at a distance of about four feet from the floor. Cyanogas A Dust was applied with the original pattern Cyanogas Foot-Pump Duster. After the products had been applied the hut was left alone for six hours. The results of these experiments are given in Table VII :—

TABLE VII.

Experiments with a hut not capable of being made even very partially air-tight.

Number of experiment.	Mean temperature, °C.	Average relative humidity, per cent.	Quantity of product applied in g. per 1,000 cu. ft.	Period of exposure, hours.	RESULTS.*	
					Rats.	Fleas.

(i) Calcid.

SERIES IX-A.

2	29·3	51·5	400	6	12/12	24/24
5	29·0	59·0	400	6	12/12	22/22
8	30·5	..	300	6	12/12	24/24
9	29·7	63·0	300	6	12/12	24/24
10	30·5	75·0	200	6	12/12	24/24
11	30·7	74·0	100	6	11/12	23/23
12	30·0	62·5	100	6	12/12	24/24
13	30·5	66·0	100	6	12/12	24/24
14	29·7	66·0	50	6	8/12	24/24
15	30·5	67·0	75	6	11/12	23/23
17	29·7	70·0	75	6	11/12	24/24

* The numerators denote the number of deaths and the denominators the number of animals used.

TABLE VII—*concld.*

Number of experiment.	Mean temperature, °C.	Average relative humidity, per cent.	Quantity of product applied in g. per 1,000 cu. ft.	Period of exposure, hours.	Results.*	
					Rats.	Fleas.

(*ii*) *Cyanogas A Dust.*

SERIES IX-B.

1	31·7	63·0	800	6	12/12	24/24
2	29·4	63·0	600	6	11/12	24/24
3	30·5	63·0	800	6	12/12	24/24
7	30·7	70·0	400	6	9/12	22/22
8	29·5	69·0	300	6	12/12	23/23
9	31·0	66·0	200	6	8/12	23/24
10	31·0	68·0	300	6	9/12	24/24
11	31·0	64·0	400	6	12/12	24/24

*The numerators denote the number of deaths and the denominators the number of animals used.

Of Calcid 100 g. per 1,000 cu. ft. of space were found to be enough to kill all the rats and the fleas under the conditions of this test, but of Cyanogas A Dust about 400 g. per 1,000 cu. ft. were required to achieve the same lethal effect. Theoretically 200 g. of Cyanogas A Dust should have proved adequate, since its content of HCN is half that of Calcid. The discrepancy as hinted before is due to the fact that Calcid gives out HCN twice as fast as Cyanogas A Dust and the very powerful Calcid Blower permits of very quick application of this product with the result that a lethal concentration of the gas is quickly reached although the gas keeps escaping from the hut all the time. Slower evolution of gas and a small applicator were handicaps in the case of Cyanogas A Dust.

Experiments were conducted to measure the actual concentration of HCN in the hut at different intervals after the fumigant had been applied. For this purpose two tubes were introduced into the hut through a side wall, one about eight feet above the floor and the other about one foot from the floor. Samples were aspirated from these tubes simultaneously at stated intervals as described above under ' the measurement of the evolution of gas, etc. ' The results obtained are given in Table VIII, in terms of samples simultaneously drawn from each of the two tubes at stated intervals :—

TABLE VIII.

Measurement of the concentration of HCN gas at stated intervals in hut experiments.

Fumigant.	Quantity introduced for 1,000 cu. ft.	Theoretical yield of HCN expressed as parts per million.	Mean temperature.	Relative humidity.	AVERAGE HCN CONCENTRATION EXPRESSED AS PARTS PER MILLION AND AS PERCENTAGES OF THE THEORETICAL CONTENT. INTERVAL AFTER THE INTRODUCTION OF THE FUMIGANT INTO THE HUT.									
					3 minutes.	8 minutes.	15 minutes.	22 minutes.	30 minutes.	36 minutes.	43 minutes.	50 minutes.	60 minutes.	120 minutes.
Cyanogas A Dust.	400 g.	2,930	30·7°C.	70%	715 (25%)	605 (21%)	456 (16%)	375 (13%)	303 (10%)	238 (8%)	191 (7%)	175 (6%)	159 (5%)	72 (2·7%)
Calcid ..	300 g.	4,400	29·7°C.	63%	2,014 (46%)	1,497 (34%)	1,176 (27%)	894 (21%)	627 (15%)	.. ..	502 (12%)	372 (9%)	231 (5%)	82 (2%)

It was unfortunate that the amounts of the fumigants used for these experiments did not have an equal content of HCN and the experiments could not be repeated as the hut was pulled down. In spite of this defect the experiments do roughly indicate that, while in the case of Calcid as much as 46 per cent of its total content of HCN was actually present in the hut after 3 minutes of its application, in the case of Cyanogas A Dust the concentration never reached higher than 25 per cent of its content of HCN.

DISCUSSION.

1. Three proprietary cyanide preparations, Calcid Briquettes, Cyanogas A Dust, and Cymag were tested to determine their relative value for the extermination of rats and fleas as a plague-preventive measure under Indian conditions. The Indian representatives of the makers were agreeable to such comparative tests being instituted and published.

2. It was found that both Calcid and Cyanogas A Dust on being blown into air yield their total theoretical content of HCN, while Cymag yields only about half of its theoretical content under the same conditions. The theoretical HCN content of Calcid is 46·80 per cent of its weight, of Cyanogas A Dust 23·31 per cent, and of Cymag 20 per cent.

3. Calcid evolves HCN gas twice as fast as Cyanogas A Dust and four times as fast as Cymag. In a closed space the maximum yield in the case of Calcid is reached in fifteen minutes, while the maximum yield in the case of Cyanogas A Dust and Cymag is reached in 30 and 60 minutes respectively. This very marked difference in the rate of evolution of HCN becomes a factor of great importance when these products are applied to Indian huts which cannot be made air-tight even partially. The reaching of a lethal concentration of HCN inside such huts is conditioned by the rate of evolution of the gas from the product and the rate of dissipation of the gas from the hut. The rate of dissipation of the gas from a given hut being constant the product which evolves the gas more rapidly would reach a lethal concentration with a smaller quantity. Thus, much smaller quantities of Calcid than those of Cyanogas A Dust, after allowing for the difference in the HCN content of these two products, are required for producing effective lethal effects under the conditions of hut disinfestation. It is however to be noted that in the case of dwellings which can be rendered reasonably air-tight the quantities of Calcid and Cyanogas A Dust needed are proportional to their HCN content.

4. For the disinfestation of rat burrows Cyanogas A Dust, when applied with the new pattern Cyanogas Foot-Pump Duster, fitted with the ' cut-out' device which permits of air being blown in independently of the powder, is approximately as effective as Calcid. But Calcid has one distinct advantage over Cyanogas A Dust in that, it is supplied in the form of small tablets of a given weight. The tablets have grooves pressed in them, which permit of their being easily broken into two or four equal parts. This makes the accurate application of small doses to rat burrows a matter of great ease. One has only to break the Calcid Briquette into required portions to get the correct dose. In the case of Cyanogas A Dust which is a powder the application of a correct dose to a rat burrow is a matter of conjecture. One

cannot carry in the field small pre-weighed quantities of this powder and put each small dose into the jar of the Cyanogas Foot-Pump Duster as required because the pump does not work efficiently unless the jar contains a largish amount of powder in it. Under the conditions the quantity of the powder applied is attempted to be controlled by the number of the strokes of the piston given. This achieves only a very rough control over the quantity applied.

Cymag was found to be quite ineffective under the conditions of the experiment described. In an experiment in which 2·5 g. of Calcid and 9·5 g. of Cyanogas A Dust were found sufficient, as much as 120 g. of Cymag failed to kill all the animals.

5. The Calcid Blowers are unquestionably superior to the Cyanogas Foot-Pump Dusters. They are much easier to operate and they produce a much more powerful blast. There is the further advantage that three different sizes of these Calcid Blowers are available in the Indian market to suit different sized operations.

The original pattern Cyanogas Foot-Pump Duster *without* the 'cut-out' device is particularly ineffective for rat-burrow fumigation. It does not carry the powder far enough into the burrow, with the result that wastefully large quantities of the dust have to be used to be certain of getting lethal concentration of HCN all through a longish burrow. In an experiment 11·8 g. of the dust when applied with the new pattern Cyanogas Foot-Pump Duster with the 'cut-out' device were found enough, more than 122·0 g. of the Dust were needed when applied with the original pattern, Cyanogas Foot-Pump Duster *without* the 'cut-out' device. With a duster with this device, after the necessary amount of the Dust has been applied to the burrow, air can be blown in to carry the Dust to the farthest points of the burrow.

Summary.

Experiments are described to determine the relative value of three proprietary cyanide preparations, Calcid, Cyanogas A Dust, and Cymag for plague control work under Indian conditions.

A FURTHER EXPERIMENT ON THE VALUE OF CALCIUM LACTATE FOR INDIAN CHILDREN.

BY

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AND

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It was observed in the Laboratories (Aykroyd and Krishnan, 1937) that the nutritive value of the poor South Indian diet for rats is increased by the addition of calcium lactate. This observation was followed up by an experiment on nursery school children aged 3 to 6 (Aykroyd and Krishnan, 1938) in which it was shown that young children given 0·5 g. of calcium lactate daily gained more rapidly in height and weight during a 5 to 6-month period than children not receiving this supplement. The present experiment, essentially similar in principle, was carried out to discover whether calcium lactate could produce the same effect on older children. Another object was to determine the relative value of calcium lactate and skimmed milk as supplements.

The investigation took place in a day school about three miles distant from the Laboratories attended by some 150 boys with ages ranging from 6 to 12, the average age being about 9. The boys were Badagas and natives of the Nilgiri Hills, consuming a diet based largely on rice and containing no milk and only small amounts of vegetables. Their state of nutrition was in general poor.

The school was divided into three groups, roughly equal as regards size and age, by working down the school list, class by class, and assigning the boys in sequence to groups I, II, and III. The groups were given the following daily supplements :—

    Group   I : (Control) .. A peppermint sweet.
    Group II :   ,,   .. A peppermint sweet + 1 gramme of calcium
                                  lactate.
    Group III :   .,   .. 8 oz. (about 250 c.c.) of liquid skimmed milk,
                                  reconstituted from 30 g. of milk powder.

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The peppermints were given for psychological reasons. Considerable experience has taught us that it is advisable, in carrying out school feeding experiments, to give all the children, including the control groups, some supplement. Otherwise the controls will feel neglected.

The experiment lasted 11 weeks, during which the supplements were given on 49 days. The children were weighed and measured at the beginning and end of the experimental period. Allowing for absentees, groups I. II. and III included respectively 42, 46, and 43 boys. Initial mean heights and weights in the three groups were approximately equal and analysis showed that the small differences in the initial means were not statistically significant.

The increments in weight and height in the three groups are compared in Tables I, II, and III, which include the necessary statistical constants.

TABLE I.

Groups I and II.   Increments and statistical constants.

	INCREMENT OF WEIGHT, LB.		INCREMENT OF HEIGHT, INCHES.	
	GROUP I.	GROUP II.	GROUP I.	GROUP II.
	Control.	Calcium lactate.	Control.	Calcium lactate.
Number in sample	42	46	42	46
Mean of sample	−0·02	+0·80	+0·42	+0·63
Standard deviation of sample ..	0·8256	1·393	0·209	0·435
Difference between two means ..	0·82		0·21	
Standard error of difference ..	0·2417		0·0718	
Difference—				
Standard error of difference ..	3·39		2·92	
Significance	Significant.		Significant.	

TABLE II.

Groups I and III. Increments and statistical constants.

	INCREMENT OF WEIGHT, LB.		INCREMENT OF HEIGHT, INCHES.	
	GROUP I.	GROUP III.	GROUP I.	GROUP III.
	Control.	Skimmed milk.	Control.	Skimmed milk.
Number in sample	42	43	42	43
Mean of sample	—0·02	1·35	0·42	0·59
Standard deviation of sample ..	0·8256	1·415	0·209	0·279
Difference between two means ..	1·37		0·17	
Standard error of difference ..	0·25		0·0535	
Difference—				
Standard error of difference ..	5·48		3·18	
Significance	Significant.		Significant.	

TABLE III.

Groups II and III. Increments and statistical constants.

	INCREMENT OF WEIGHT, LB.		INCREMENT OF HEIGHT, INCHES.	
	GROUP II.	GROUP III.	GROUP II.	GROUP III.
	Calcium lactate.	Skimmed milk.	Calcium lactate.	Skimmed milk.
Number in sample	46	43	46	43
Mean of sample	0·80	1·35	0·63	0·59
Standard deviation of sample ..	1·393	1·415	0·435	0·279
Difference between two means ..	0·55		0·04	
Standard error of difference ..	0·298		0·077	
Difference—				
Standard error of difference ..	1·85		0·52	
Significance	Almost significant.		Not significant.	

DISCUSSION.

The results show that weight and height increments in both the calcium lactate and the skimmed milk groups were significantly greater than in the control groups. While the control group did not increase in weight, the mean increases in groups II and III were 0·8 lb. and 1·35 lb. respectively. The difference between the calcium lactate and skimmed milk groups as regards weight increase falls just short of statistical significance. There was no significant variation in respect of height increments in these groups. It must be remarked, however, that the general condition of the children receiving milk showed an improvement which was less evident in the calcium lactate groups—an improvement which we have observed again and again when poor Indian children are given extra milk. It may be concluded that, while calcium lactate is of benefit under the circumstances, it is naturally a less valuable supplement than milk. Similar observations have been made on groups of rats (Aykroyd and Krishnan, 1937).

In the experiment described the period of extra feeding was somewhat short; owing to a combination of circumstances it was impossible to continue for a longer period. The experiment was, however, of value in confirming the results of the earlier experiment in which a supplement of calcium lactate was found to have a beneficial effect on children in a nursery school. Half to one gramme of calcium lactate could be provided to a child at a cost of about 1 anna per month.

SUMMARY.

1. South Indian day school children given a supplement of 1 gramme of calcium lactate for a period of about 11 weeks showed significantly greater height and weight increments than children not receiving calcium.

2. In a similar group receiving skimmed milk height and weight increments were also significantly in excess of those recorded in the control group. The skimmed milk group showed more improvement in general condition than the calcium lactate group. Weight increase in the former was somewhat larger than in the latter but the difference was not quite significant.

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NOTE ON THE ASSIMILATION OF CAROTENE BY RATS FROM A FAT-FREE DIET.

BY

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IT was previously reported (Wilson, Ahmad and Majumdar, 1936) that carotene fed as a colloidal watery solution was absorbed by rats. Although the absorption was poor compared with that of other groups receiving supplements of meat and fat, it nevertheless gave the animals adequate protection against vitamin-A deficiency. It was also shown that the absorption of carotene was definitely better when the hydrocarbon was fed dissolved in oil. Basu (1937) has reported that rats receiving a fat-free diet deficient in vitamin A failed to respond to curative doses of vitamin A or carotene. Since in the experiments referred to above only a comparative study of the absorption of carotene by animals fed on a synthetic diet containing various supplements was made, care was not taken to remove the last traces of fat from the basal diet, which was composed of heated rice starch 68 parts, casein (B.D.H.) 18 parts, yeast 7 parts, and salt mixture 7 parts. It was therefore thought not unlikely that the little fat ordinarily present in the diet might have helped in the absorption of the pigment. The present experiment was undertaken to settle this point.

The ordinary stock diet used for rats in the Laboratories is rich in vitamins and carotene and experience has shown that animals given vitamin-A deficient diets after previous feeding on the stock diet, do not show signs of vitamin-A deficiency for long periods. For the present experiment, therefore, a modified stock diet was given to the female rats during pregnancy and lactation and to their offspring during the period just after weaning.

The diet was as follows :—

	Parts.
Wheat (atta)	18·0
Lean meat	2·0
Gingelly oil	1·0
Colocasia antiquorum	2·6
Potatoes	2·7
Plantain fruit	4·0
Dhal arhar	1·0

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When the young rats were about 40 to 50 grammes in weight, they were given a synthetic vitamin-A deficient diet. This diet was extracted with ether and contained less than 0·04 per cent fat.

Its composition was as follows :—

					Parts.
Starch	..	..	..	..	65
Casein	..	..	..	..	20
Dried yeast	..	..	..	..	8
Salt mixture	..	..	..	..	7

After 6 to 7 weeks on this diet, most of the animals began to show eye changes characteristic of vitamin-A deficiency. They were then given varying doses of colloidal carotene in 2 per cent glucose solution. Colloidal carotene solution was prepared by evaporating off the acetone from a solution of carotene in that solvent to which a little 2 per cent glucose water was initially added. The doses of carotene ranged from 7·2 μg. to 12·0 μg. per day. Improvement of xerophthalmia occurred in 4 to 5 days. On discontinuing the supplement, the eye conditions reappeared within about a week. Of 55 rats with xerophthalmia given carotene in colloidal solution, 48 were cured. Of the rats treated, 20 were animals which developed xerophthalmia a second time after the carotene supplement was stopped.

Storage of vitamin A.

Two groups of four rats with signs of vitamin-A deficiency were fed 12·0 μg. and 7·2 μg. respectively daily for a week. The rats were then killed and the vitamin-A content of their livers determined spectrophotometrically according to the method described by De (1937). The band at 328 mμ. was taken as characteristic of vitamin A. A similar determination of the vitamin-A content of a control group of six deficient animals was made.

The results are recorded below :—

	Carotene per rat per day, μg.	Total carotene fed to the group, μg.	Vitamin A per g. of liver, μg.
Group I.			
Rat No. 12 ..	12·0		
,, 20 ..	12·0	336·0	0·8
,, 30 ..	12·0		
,, 34 ..	12·0		
Group II.			
Rat No. 13 ..	7·2		
,, 27 ..	7·2	201·6	0·5
,, 31 ..	7·2		
,, 36 ..	7·2		

The livers of six rats, killed when suffering from eye changes after a period on the vitamin-A deficient diet, were found to contain 0·18 μg. of vitamin A per gramme of liver. It thus appears that little or no storage of vitamin A in the livers takes place when small amounts of carotene (7 μg. to 12 μg. per day) are given to a depleted rat.

SUMMARY.

Carotene fed in colloidal solution in water can be absorbed by rats even when the diet contains less than 0·04 per cent fat.

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THE URINARY EXCRETION OF NICOTINIC ACID.

BY

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INTRODUCTION.

IT is now well established that nicotinic acid and its amide have a curative action in pellagra and that they are essential components of the human diet. But little is known regarding the metabolism of nicotinic acid in human beings. The present paper deals with this aspect of the problem.

In a recent communication from these Laboratories (Swaminathan, 1938) a chemical method was described for the quantitative estimation of nicotinic acid in foodstuffs and blood. It was found that the pellagra-preventive (P-P) values of foodstuffs reported by previous workers and based on biological investigations (Sebrell, 1934) corresponded roughly with their nicotinic-acid content. Investigations proceeding in these Laboratories have shown that milled rice, as compared with certain other cereals, is not a good source of the vitamin. Hence it appears probable that diets based largely on milled rice may be partially deficient in nicotinic acid.

As a preliminary approach to this question, an investigation of the excretion of nicotinic acid by various groups has been undertaken. The response to test doses, which may indicate the degree of 'saturation' present, was also studied. The method followed is analogous to that of Harris and his co-workers for detecting partial deficiency of vitamins B_1 and C (Harris and Ray, 1935 ; Harris and Leong, 1936 ; Harris, Leong and Ungley, 1938), by determining the excretion of these factors under ordinary circumstances, and before and after the administration of test doses.

METHODS.

Ingested nicotinic acid or its amide may appear in the urine either as nicotinic acid (I), nicotinamide (II), nicotinyl-glycine (nicotinuric acid) (III), trigonellin (IV) and the codehydrogeneses, 1 and 2 (Ackermann, 1912 ; Komori and Sendju, 1926 ; von Euler *et al.*, 1938).

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Vilter *et al.* (1938) have proposed a method for the estimation of nicotinic-acid conjugates in urine using 2 : 4-dinitrochlorobenzene as a reagent. They found that nicotinic acid gave a purple colour and the amide a red colour, but they made no attempt to convert the amide into acid ; further it is not known what colours would be given by nicotinyl-glycine and the codehydrogeneses. It has also been shown previously (Swaminathan, *loc. cit.*) that the amide gives only about half as much colour as the acid with the cyanogen-bromide-aniline reagent. It can also be seen from Table I that the values obtained for the nicotinic-acid content of urine before hydrolysis are much lower than those obtained after hydrolysis, which is evidently due to the presence of nicotinic-acid derivatives. Hence an accurate and complete estimation of nicotinic acid in urine involves the conversion of its derivatives into the free acid before any colorimetric test can be applied. These can be readily hydrolysed to nicotinic acid by boiling, with mineral acids, or alkalis (Ackermann, *loc. cit.* ; Swaminathan, *loc. cit.* ; von Euler *et al.*, *loc. cit.*). Previously, when dealing with foodstuffs and blood, 5 per cent hydrochloric acid was used. In
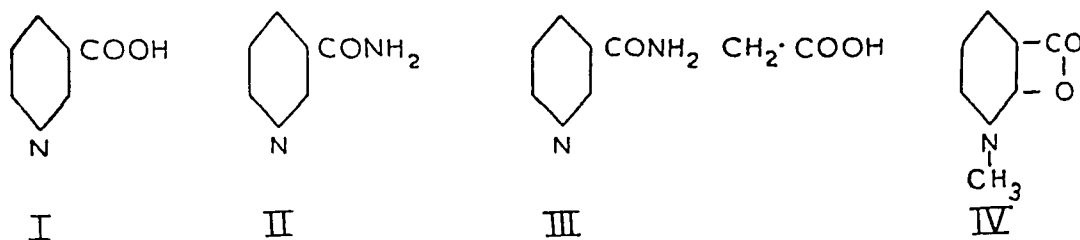
the present investigation 8 per cent sodium hydroxide has been employed. Equally good results are obtained with the former in the examination of urine, but the latter has slight advantages in that it removes the ammonia formed during hydrolysis. After hydrolysis the solution is decolorized with charcoal (Norit) in a slightly alkaline medium.

The colorimetric method used is a modification of that previously described by the author (Swaminathan, *loc. cit.*) for the estimation of nicotinic acid in foodstuffs and blood. Trigonellin is not estimated by the method.

PROCEDURE.

Reagents required :—

(1) Standard nicotinic acid (strong) 1 ml. = 1 mg. nicotinic acid dissolved in $\frac{N}{100}$ HCl, and kept in a refrigerator.

(2) Standard nicotinic acid (dilute) 1 ml. = 10 μg. nicotinic acid, prepared fresh as required by diluting 0·5 ml. of solution (1) to 50 ml. after neutralizing with 0·5 ml. of $\frac{N}{100}$ caustic soda.

(3) Aqueous cyanogen bromide : This is prepared fresh by decolorizing in the cold a saturated aqueous solution of bromine by the gradual addition of 10 per cent sodium cyanide.

(4) Aqueous aniline solution, prepared fresh by dissolving 2 ml. of aniline in 80 ml. of distilled water and diluting to 100 ml.

(5) Phosphatic buffer pH 7 (Clark and Lubs).

(6) Sodium hydroxide 40 per cent.

To 50 ml. of urine are added 12 ml. of 40 per cent caustic soda and the mixture boiled under reflux for 3 hours. In preliminary experiments using normal urines a period of $1\frac{1}{2}$ to 2 hours was found to be sufficient to complete hydrolysis. Urines collected after test doses have been given may conceivably contain appreciable amounts of nicotinyl-glycine, which might require about 3 hours for hydrolysis (Ackermann, *loc. cit.*). The mixture, after cooling, was neutralized with concentrated hydrochloric acid and then made slightly alkaline (about $\frac{N}{10}$ NaOH) by the addition of 0·5 ml. of 40 per cent caustic soda. Generally a precipitate forms at this stage. This is removed by centrifuging and washed once with 20 ml. to 30 ml. of water. The combined centrifugates (about 90 ml. to 100 ml.) are boiled for about 20 to 30 minutes with 2 g. of charcoal. This results in the concentration of the solution to about 60 ml. It is filtered hot, and the charcoal washed by boiling with 20 ml. to 30 ml. of distilled water, containing 2 to 3 drops of 40 per cent caustic soda. The mixed filtrate, after cooling, is brought to pH 7 with dilute hydrochloric acid, filtered, and made up to a convenient volume. In general, the solutions are practically colourless, but occasionally they may have a light yellow tinge when a blank estimation is being carried out.

The colorimetric method previously described has been slightly modified by the use of (1) 2 ml. of phosphatic buffer of pH 7 to stabilize the reaction, (2) 8 ml. of cyanogen-bromide solution instead of 4, the former quantity having been found to give the maximum colour with 10 μg. to 50 μg. of nicotinic acid.

Aliquots of the extract and the standard nicotinic acid (10 μg. to 50 μg.) are measured out and each diluted to 10 ml. Two ml. of phosphate buffer of pH 7 are added followed by 5 drops of aqueous aniline. Eight ml. of cyanogen-bromide solution are then added to the solutions, the mixture being shaken as the addition is being made. A bright yellow colour develops and reaches its maximum intensity within one minute. Five ml. of aqueous aniline are now added to each. The colours, which remain stable for over thirty minutes, are compared in a colorimeter immediately.

Notes :—

1. In the case of urines collected after test doses, which are likely to be rich in nicotinic acid, the solution before decolorization with charcoal should be diluted to about twice the volume indicated above.

2. In the case of urines containing small amounts of nicotinic acid the volume of the final solution should be such that the concentration of nicotinic acid is at least 0·5 μg. per ml.

3. In certain cases in which the test solutions were of yellow colour, one estimation was carried out with each solution by the method described, but without the addition of cyanogen bromide, distilled water being added instead. The values so obtained for the 'blanks' were subtracted from the total values to get the true values.

Recovery of nicotinic acid added to urines in the form of nicotinic acid and its amide.—Different known amounts of nicotinic acid and its amide were added to known volumes of urine. The recovery was good in all the cases. The results are given in Table II. All the specimens of urine except No. 1 were collected after test doses.

TABLE I.

The estimation of nicotinic acid in urine before and after hydrolysis (urine samples collected after test doses).

Sample number.	Before hydrolysis (mg.).	After hydrolysis (mg.).
1	12·32	21·59
2	5·04	10·28
3	7·12	14·68
4	9·91	15·30
5	7·78	15·30
6	46·93	67·96

TABLE II.

Recovery of nicotinic acid added to urine.

Experiment number.	Urines with and without added nicotinic acid and its amide.	Total nicotinic acid (mg.).	Recovery, per cent.
1	Urine 50 ml.	0·055	
	Urine 50 ml. + 0·05 mg. nicotinic acid + 0·05 mg. nicotinamide.	0·151	
	Recovery	0·096	96
2	Urine 50 c.c.	0·195	
	Urine 50 c.c. + 0·1 mg. nicotinic acid + 0·1 mg. nicotinamide.	0·388	
	Recovery	0·193	97

TABLE II—*concld.*

Experiment number.	Urines with and without added nicotinic acid and its amide.	Total nicotinic acid (mg.).	Recovery, per cent.
3	Urine 50 c.c.	0·314	
	Urine 50 c.c. + 0·1 mg. nicotinic acid + 0·1 mg. nicotinamide.	0·508	
	Recovery	0·194	97
4	Urine 50 c.c.	0·199	
	Urine 50 c.c. + 0·25 mg. nicotinic acid + 0·25 mg. nicotinamide.	0·715	
	Recovery	0·516	103
5	Urine 50 c.c.	0·197	
	Urine 50 c.c. + 0·25 mg. nicotinic acid + 0·25 mg. nicotinamide.	0·750	
	Recovery	0·553	110
6	Urine 50 ml.	1·210	
	Urine 50 ml. + 0·5 mg. nicotinic acid + 0·5 mg. nicotinamide.	2·160	
	Recovery	0·950	95

The excretion of nicotinic acid.—The present investigation was carried out on 34 subjects, 10 laboratory workers and 24 hospital patients in Coonoor. The total nicotinic acid excreted was estimated in two 24-hour samples of urine before and after the ingestion of 100 mg. of nicotinic acid. This dose was chosen, because it was found to be very rarely associated with any unpleasant reactions (e.g., headache and flushing). Satisfactory response was obtained in preliminary trials in normal subjects. During the experimental period the hospital patients were on a diet of parboiled milled rice with approximately six ounces of vegetables and pulses daily, and containing no milk or flesh foods. This corresponds roughly with the diet consumed by the poorer classes in the district in which the

subjects were living. The normal controls consumed their usual diets, avoiding the use of any vitamin preparations.

Table III shows that subjects 1 to 4, whose diets were based on whole wheat with liberal amounts of milk and/or flesh foods excreted on an average 6·8 mg. of nicotinic acid daily and 27·9 mg. following the test dose of 100 mg. The same results are shown graphically in the Chart. Subjects 5 to 10, workers in the Laboratories, whose diets were based on milled rice with varying amounts of milk, vegetables, and flesh foods, excreted on an average 3·2 mg. nicotinic acid daily and 19·7 mg. following the test dose.

The first group of hospital patients included 5 cases of peripheral neuritis and 3 cases of stomatitis, all of dietetic origin. The peripheral neuritis in these cases was due probably to vitamin-B_1 deficiency occurring as a result of consuming a diet chiefly composed of milled rice. Presumably such a diet is also partially deficient in nicotinic acid. The stomatitis cases were similar to those described by Aykroyd and Krishnan (1936) and in each case there was definite improvement on nicotinic-acid therapy. The average daily nicotinic-acid excretion in the urine was 1·15 mg. and 5·1 mg. following the test dose.

TABLE III.

Excretion of nicotinic acid before and after administration of test doses.

Group and number.	Diagnosis.	Initial excretion in 24 hours (mg.).	Response to a test dose of 100 mg. in 24 hours (mg.).
I. LABORATORY WORKERS (NORMAL SUBJECTS).			
(a) *Wheat-eaters :*			
1. K. L. S.	....	8·29	37·85
2. R. P.	....	8·88	26·67
3. N. S.	....	5·13	26·94
4. D. S.	....	4·76	20·03
AVERAGE	....	6·77	27·87

TABLE III—*contd.*

Group and number.		Diagnosis.	Initial excretion in 24 hours (mg.).	Response to a test dose of 100 mg. in 24 hours (mg.).
(b) *Rice-eaters :*				
5. K. V. G.	.. ..	....	4·21	18·22
6. P. ..	.. ..	....	4·12	20·90
7. B. ..	.. ..	....	3·44	17·25
8. B. M. M.	.. ..	....	2·59	26·15
9. M. S.	.. ..	....	2·46	21·58
10. S. M.	.. ..	....	2·06	14·06
AVERAGE	..	....	3·15	19·69
II. HOSPITAL PATIENTS.				
(a) *With clinical conditions due to dietary deficiency :*				
11. L. ..	.. ..	Peripheral neuritis.	0·91	13·61
12. A. ..	.. ..	,, ,,	1·21	4·60
13. D. ..	.. ..	,, ,,	1·24	4·59
14. K. ..	.. ..	,, ,,	0·42	4·05
15. M. ..	.. ..	,, ,,	1·05	2·97
16. C. ..	.. ..	Stomatitis.	2·20	6·11
17. P. ..	.. ..	,,	0·88	1·86
18. R. ..	.. ..	,,	1·32	2·97
AVERAGE	..	....	1·15	5·10

TABLE III—*concld.*

Group and number.	Diagnosis.	Initial excretion in 24 hours (mg.).	Response to a test dose of 100 mg. in 24 hours (mg.).
(b) *Without any clinical signs of dietary deficiency :*			
19.　C.　..	Injury to heel	1·65	10·63
20.　K.　..	Injury to head	1·09	10·53
21.　Ka..　..	Sprained ankle	1·02	6·93
22.　R.　..	Ulcer on leg	1·38	6·52
23.　K.　..	Injury to thigh	1·16	2·08
24.　V.　..	Warts	0·97	1·88
25.　Va..　..	Scabies	2·06	24·70
26.　I.　..	Rheumatism	0·90	23·69
27.　R.　..	,,	2·91	15·18
28.　S.　..	Syphilis	1·92	14·96
29.　An..　..	Diarrhœa	1·43	10·61
30.　C.　..	,,	1·18	8·94
31.　H.　..	,,	0·70	7·07
32.　R.　..	Asthma	0·71	7·03
33.　A.　..	Recovering from slight fever.	1·64	5·03
34.　K.　..	Gastritis	0·58	1·70
AVERAGE	....	1·33	9·84

The other hospital patients were a miscellaneous group, suffering from conditions apparently unrelated to diet deficiency. It can be taken for granted that the diets consumed by this group previous to admission were defective in various respects, though perhaps a little better than those consumed by group I. The

CHART.
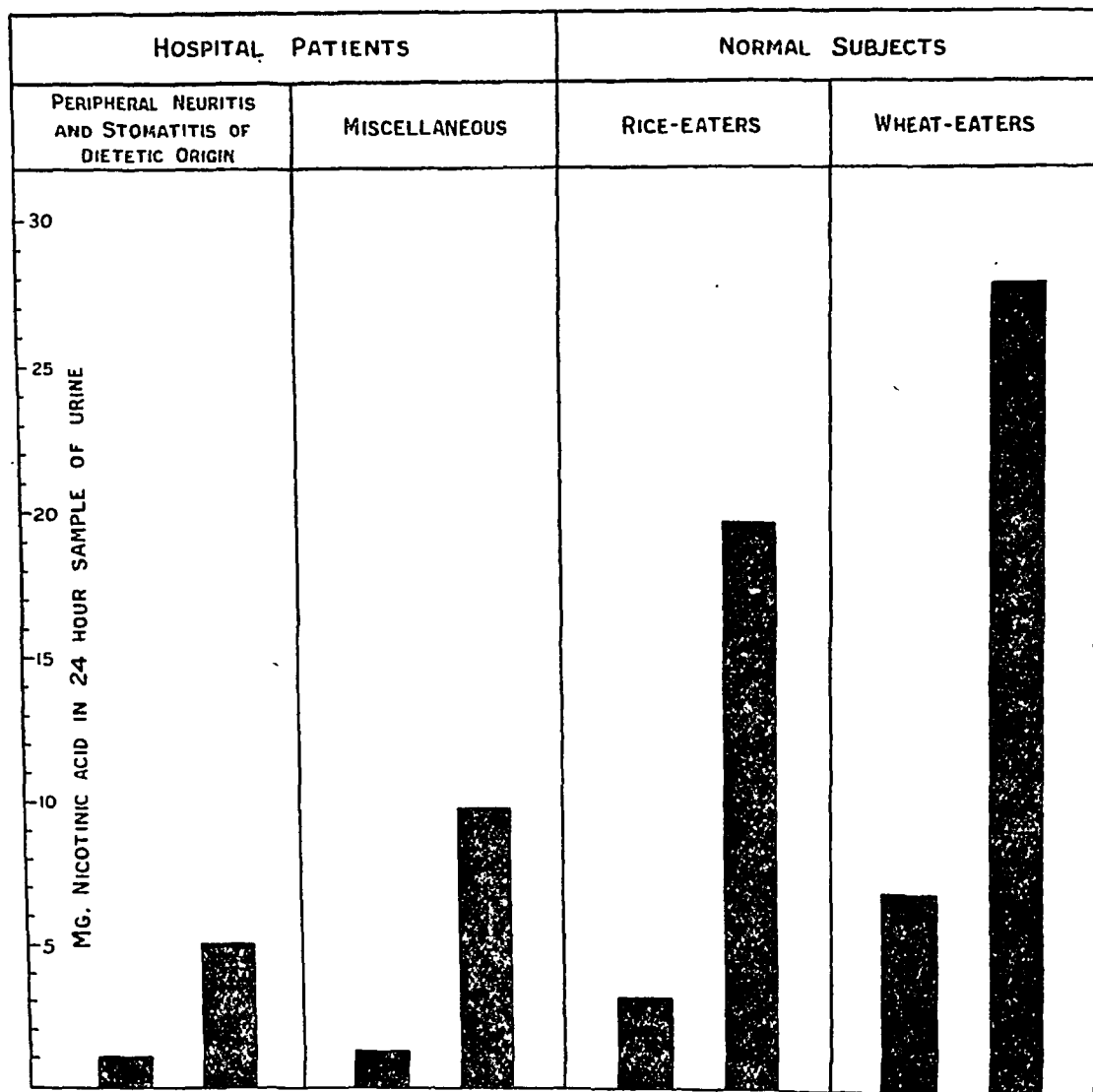
Excretion of nicotinic acid by various groups before and after a test dose of 100 mg. nicotinic acid.

average nicotinic-acid excretion in this group was 1·3 mg. daily and 9·9 mg. following the test dose.

'*Saturation*' *experiments with nicotinic acid.*—A further series of experiments was undertaken to study the point of 'saturation' with nicotinic acid in one

normal subject (M. S.) consuming a diet composed of rice, pulses, and vegetables, and including about 500 ml. of milk daily. A similar experiment was conducted for a shorter period on a subject (M. C.) suffering from stomatitis, consuming a rice diet without milk. The results are set out in Table IV :—

TABLE IV.

Nicotinic acid ' saturation' experiments.

Name.	Diagnosis.	Days.	Dose (mg.).	Excretion following test dose of 100 mg. (mg.).
M. S. ...	Normal	1	..	2·46
		2	..	2·74
		3	..	2·68
		4	100	21·58
		5	100	15·60
		6	100	21·59
		7	100	10·28
		8	100	9·17
		9	100	10·20
		10	100	14·68
		11	100	11·16
		12	100	9·85
		13	100	9·53
		14	100	15·30
		15	100	9·59
		16	100	15·30
		17	100	9·23
		18	500	67·96
		19	25	6·82
		20	..	3·04
		21	..	2·37
		22	..	2·61

Table IV—*concld.*

Name.	Diagnosis.	Days.	Dose (mg.).	Excretion following test dose of 100 mg. (mg.).
		1	..	2·20
		2	100	6·11
		3	200	8·71
M. C. ..	Stomatitis	4	400	10·38
		5	400	10·25
		6	400	18·80

The average initial excretion of the normal subject (M. S.) was 2·63 mg. daily. It may be roughly estimated that in the case of this subject the ordinary daily intake would be of the order of 20 mg., so that excretion represented about 12 per cent of intake. During the daily ingestion of 100 mg. for a period of 14 days, the urinary excretion ranged from 10 mg. to 21 mg., the average for the period being 13·15 mg. On the 18th and 19th days, when 500 mg. and 25 mg. of nicotinic acid were taken, the urinary excretion was 67·96 mg. and 6·82 mg. respectively. During the next 3 days, when the test doses were discontinued, the excretion fell to the low initial level, the fall taking place within 24 hours. It therefore appears that a fraction of the intake—about 13 per cent on the average—is excreted daily irrespective of the amount ingested and the period of administration. What happens to the larger fraction which is not excreted, and how much is converted into trigonellin, are problems for further study.

The behaviour of the subject suffering from stomatitis as regards the excretion of nicotinic acid following the administration of test doses was different from that of the normal subject. Only 2 to 4 per cent of the ingested nicotinic acid was excreted in the period of 6 days. Clinically the patient showed rapid improvement following the administration of nicotinic acid, the mouth lesions almost disappearing within 4 days.

DISCUSSION.

It will be seen from Table III that there was a wide individual variation in the initial levels of excretion of nicotinic acid in the 10 normal subjects investigated, the values ranging from 2 mg. to 8·9 mg., while the responses to the test doses varied from 12 to 29 per cent of the amounts ingested, due allowance being made for the initial excretion.

In the group of hospital patients with clinical signs of diet deficiency, the average urinary excretion before and after the test dose was only 1·15 mg. and 5·1 mg. respectively. In the other group, consisting of 16 patients without any clinical signs of dietary deficiency, though the initial level of excretion was low,

the excretory response after the test dose was on the average 9·9 mg. The results suggest that the former group of patients was more deficient in nicotinic acid than the latter.

SUMMARY.

1. An investigation of the urinary excretion of nicotinic acid has been carried out by the cyanogen-bromide-aniline method.

2. The average daily excretion in 4 groups consisting of wheat-eaters, laboratory workers consuming a fairly well-balanced diet based on rice, and hospital patients of the poorer classes with and without signs of deficiency disease, was determined, daily averages for these groups being 6·77 mg., 3·15 mg., 1·15 mg., and 1·33 mg., respectively.

3. Urinary excretion in the above groups during a period of 24 hours after a test dose of 100 mg. nicotinic acid was 27·87 mg., 19·69 mg., 5·10 mg., and 9·84 mg., respectively.

4. The administration to a normal subject of 1,925 mg. over a period of 18 days did not produce ' saturation ', excretion at all levels of intake being about 12 per cent of the amount ingested. In a case suffering from stomatitis the percentage of the intake excreted was of a much smaller order, in the region of 4 per cent.

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  and HOGBERG (1938).

INDIAN GOOSEBERRIES (*PHYLLANTHUS EMBLICA* LINN.) AS A SOURCE OF VITAMIN C.

BY

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IN a previous publication (Giri and Doctor, 1938), the results of an investigation of the vitamin-C potency of Indian gooseberries and its variation on drying were presented. It was pointed out that this fruit is the richest source of vitamin C among foodstuffs hitherto investigated. It contains about 7 mg. of vitamin C per g. of the fresh pulp. The powder obtained on drying the berries at room temperature was found to contain as much as 25 mg. of vitamin C per gramme. Thus, the vitamin can be obtained in a concentrated form as dried powder for use when fresh berries are out of season. In general, however, vegetable materials and fruits lose their vitamin-C potency on drying and storage. It was, therefore, thought desirable that the potency of the dried material should be confirmed by further tests. Since the chemical method of estimating vitamin-C content of foodstuffs is sometimes misleading, on account of the presence of substances other than vitamin C which also reduce the indophenol-dye, the results of chemical estimation of vitamin C should if possible be checked by biological tests.

The present paper describes the biological determination of the vitamin-C content of Indian gooseberries, and the effect of storage and cooking on the vitamin C present.

Indian synonyms for Indian gooseberry (Sheriff, 1869).

Hindustani and Bengali ..	..	*Amla.*
Telugu	..	*Nelli-kayi, Usirike-kaya, Amalakam.*
Canarese	..	*Nelli-kayi.*
Tamil	..	*Nelli-kayi.*
Malayalam	..	*Amalakam, Nelli-kaya.*
Guzarathi	..	*Ambala.*
Arabian	..	*Amlaj.*
Persian	..	*Amelah.*
Burmese	..	*Ziphiyu-si.*
Marathi	..	*Avala.*

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* *Lady Tata Memorial Scholar.*

BIOLOGICAL ASSAY.

The antiscorbutic potency of an aqueous extract of dried gooseberry powder was determined by the usual method based on the weight increase of young guinea-pigs. The following diets (scorbutic diets A and B) formed the basal diets in these experiments. The composition of the diets is shown below:—

Scorbutic diet A.

	Per cent.
Rice bran ..	45
Crushed oats	25
Skimmed milk powder	25
Yeast	3
Calcium carbonate	1
Sodium chloride	1

The composition of the diet is substantially the same as that of the scurvy-producing diet described by Reedman and McHenry (1938).

Scorbutic diet B.

	Per cent.
Crushed oats	62
Skimmed milk powder	30
Gingelly oil	5
Dried yeast	1
Sodium chloride	1
Cod-liver oil	1

The composition of this diet is slightly different from that of scorbutic diet A described above.

The preparation of an aqueous extract of vitamin C from Indian gooseberry powder.—The extract was obtained by extracting 2 g. of the powder, the preparation of which is described elsewhere in the present paper, with 20 ml. of water and filtering it after standing for about 10 to 15 minutes. The clear yellow filtrate which contains the vitamin was used for the experiments, after suitable dilution with water, the concentration of vitamin C being 1 mg. per ml. of the solution. The vitamin content of the extract was determined by titration with indophenol-dye, which was standardized against pure synthetic l-ascorbic acid (B.D.H.).

Two sets of experiments (A and B), each including three groups of young guinea-pigs, were set up. Each sub-group in experiment A contained six animals weighing about 190 g. to 200 g. The first group in experiment B contained six animals, and the second and third groups contained five animals in each. The following diets were given:—

EXPERIMENT A.

    GROUP I.   The scorbutic diet A alone.

    GROUP II. The scorbutic diet A + 2 ml. of gooseberry extract containing 1·9 to 2·0 mg. vitamin C.

GROUP III. The scorbutic diet A + 2 mg. of synthetic l-ascorbic acid. The acid was dissolved in water to a concentration of 2 mg. per ml.

EXPERIMENT B.

GROUP I. The scorbutic diet B alone.

GROUP II. The scorbutic diet B + 2 ml. gooseberry extract containing 5 mg. vitamin C.

GROUP III. The scorbutic diet B + 5 mg. synthetic l-ascorbic acid.

In addition each guinea-pig in experiment A was given 1 ml. cod-liver oil twice weekly by pipette into the back of its mouth. The animals were weighed twice a week.

CHART 1.

Time in days.
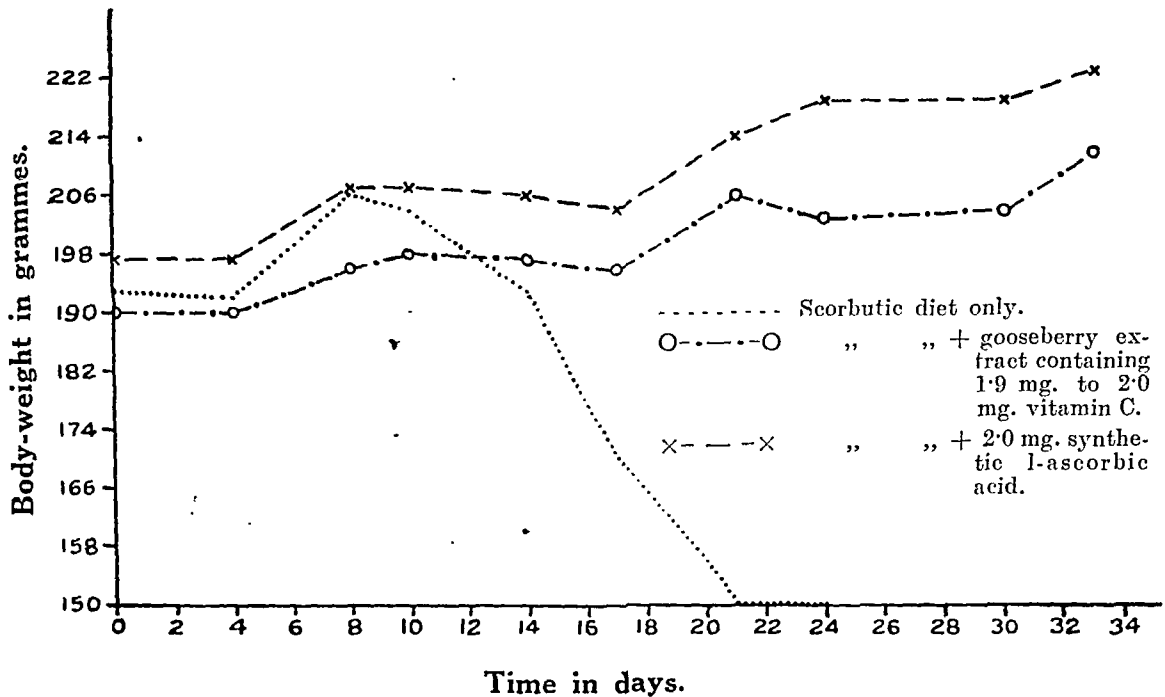
Weight curves of groups on vitamin-C deficient diet (A) and the same diet supplemented by Indian gooseberry powder extract and synthetic l-ascorbic acid.

The average increase in weight during the period of the experiments is represented in graph form in Charts 1 and 2. During the five week experimental period it was found to be 22 g. and 26 g. in groups A-II and A-III respectively and 123 g. and 170 g. in groups B-II and B-III respectively.

All the animals receiving the scorbutic diet died within four weeks, while the animals in the other two groups survived. Post-mortem examination of the guinea-pigs receiving the unsupplemented diets revealed characteristic signs of scurvy, hæmorrhages into the knee joints and adrenals. The other groups

CHART 2.
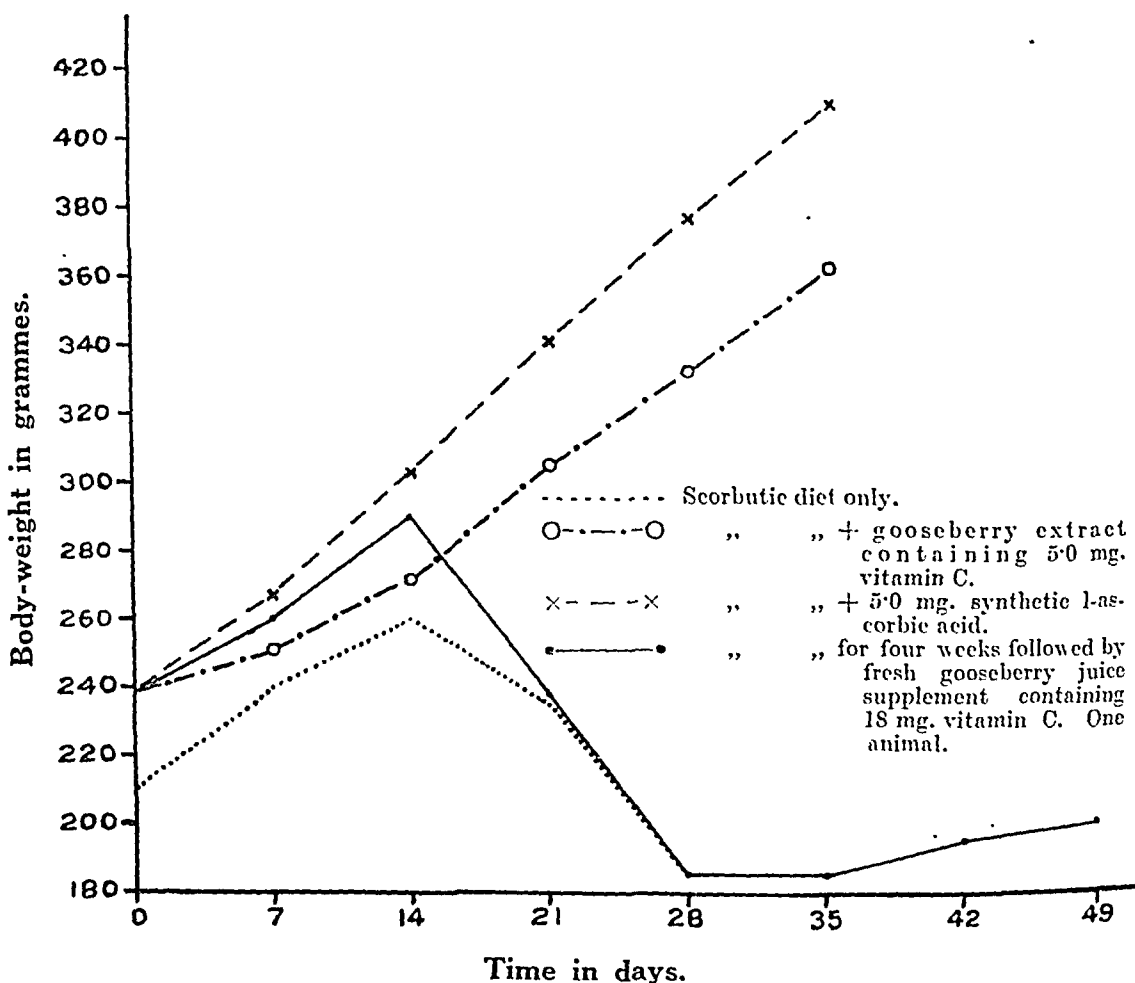
Weight curves of groups on vitamin-C deficient diet (B) and on same diet supplemented by Indian gooseberry powder extract and synthetic l-ascorbic acid.

showed no evidence of scurvy. Two animals in group B-II, however, died of broncho-pneumonia within the first week of starting the experiment.

The curative effect of gooseberry juice on animals fed on a scorbutic diet was next tried. One of the animals which showed typical signs of scurvy after

four weeks on the scorbutic diet was given 2 ml. daily of freshly prepared gooseberry juice. The juice contained 9 mg. of vitamin C per ml. Signs of scurvy disappeared and the animal increased in weight. The growth curve of this animal is shown in Chart 2.

The average increase in weight of animals given the gooseberry extract was slightly lower than that of animals given an equal amount of synthetic l-ascorbic acid. This difference may, however, be considered to lie within the limits of the accuracy of the method. Further, on comparing the results obtained in the two sets of experiments, it may be noted that the average increase in weight of the animals is not proportional to the dose of the vitamin given. In this connection the views of Coward and Kessner (1936) with regard to the degree of accuracy obtainable by this method may be quoted. 'This test is more accurate when doses are chosen which just maintain weight in the guinea-pigs than when doses are chosen which bring about a large increase in weight. The dose which will bring about a mean increase in weight of 50 g. is only double the dose which will bring about a mean increase in weight of 10 g. Thus, there is a very narrow range of effective doses for a vitamin-C test'. For all practical purposes, therefore, the reducing substance present in the fruits, as determined by the dye method, may be considered to be equivalent to vitamin C in antiscorbutic properties, and in subsequent studies on the changes in the vitamin-C content of the fruits on storage and cooking it was considered justifiable to use the dye method for its estimation.

The vitamin-C content of the tissues of the experimental guinea-pigs.—The vitamin-C content of the liver, kidney, and adrenals of the animals belonging to the various groups was determined chemically by titration with 2 : 6-dichlorophenol indophenol. Tissues for examination were removed, weighed, ground with glass-powder in the presence of 5 per cent trichloracetic acid containing 1 per cent sodium pyrophosphate, extracted three times with the acid-pyrophosphate mixture, centrifuged, and titrated with the dye. The addition of pyrophosphate to trichloracetic acid protects the vitamin against oxidation during extraction (Giri and Doctor, *loc. cit.*). The pyrophosphate was prepared according to the method described in a previous communication (Giri, 1937).

The vitamin-C contents of the liver, kidney, and adrenals of the experimental guinea-pigs in groups II and III were found to vary·between 0·096 mg. and 0·183 mg., 0·060 mg. and 0·117 mg., and 0·75 mg. and 1·3 mg., per g. respectively, while the corresponding average values for the tissues of scorbutic animals were < 0·02 mg., 0·01 mg., and 0·08 mg., per g. respectively. The vitamin was thus present in negligible amounts in the tissues of scorbutic animals.

Method of preparing powder from Indian gooseberries.—Drying is one of the methods used to preserve fruits for use out of season. Usually considerable loss of vitamin C occurs during the process of drying. The effect of drying on the vitamin-C content of gooseberries was investigated. The fruits were minced into small pieces with the aid of a household scraper, and the fresh pulp thus obtained was spread into thin layers on enamelled trays and dried under an electric fan at room temperature (20°C. to 25°C.). The material can be dried in two days. After complete drying it was finely powdered and stored in bottles with air-tight

stoppers.   The influence of sun drying on the vitamin-C potency of the material was also studied.   The results are presented in Table I :—

TABLE I.

Influence of drying on the vitamin-C content of Indian gooseberries.

Treatment.	VITAMIN C IN MG. PER 100 G.		
	Sample No. I.	Sample No. II.	Sample No. III.
1. Fresh pulp	473	611	680
2. „ „ (moisture free) ..	2,428	3,090	3,470
3. Powder obtained by drying the fresh pulp in shade at room temperature.	1,780	2,280	2,660
4. Powder obtained by drying the fresh pulp in the sun.	1,550	1,840	..

Although there is some loss of vitamin C on drying under a fan at room temperature, the powder contains as much as 18 mg. to 26 mg. of vitamin C per g. of the material.   Sun drying was found to cause much greater losses of the vitamin.   The losses were 26 and 36 to 40 per cent. respectively.   A concentrated preparation of the vitamin in the form of powder can be easily obtained from the fruits by the method described above.

Variation in the vitamin-C content of the dry powder on storage.—In view of the known tendency of some vitamin preparations to lose potency on storage, the powder obtained from gooseberries by sun drying was examined for its vitamin content during storage for varying periods at different temperatures.   The results are shown in Table II :—

TABLE II.

Vitamin-C content of Indian gooseberry powder on storage at different temperatures.

Period of storage in days.	VITAMIN C IN MG. PER 100 G. OF THE SUN-DRIED POWDER.		
	Stored in refrigerator at 0°C.	Stored at room temperature, 20°C. to 25°C.	Stored in incubator at 37°C.
0	1,380	1,380	1,380
42	1,350	1,050	762
103	1,386	950	357

The results show that the vitamin-C potency of the powder is not impaired after storage in a refrigerator for three months. The powder stored at room temperature (20°C. to 25°C.) was found to have lost about 30 per cent of the vitamin, while the sample kept at 37°C. in the incubator lost 74 per cent after three months' storage. Thus, the vitamin contained in the powder is not very stable to temperatures higher than 25°C.

The vitamin-C content of the expressed juice.—Fresh fruits were minced with a household scraper and the juice was squeezed out of the pulp in a piece of cloth. The vitamin-C content of the expressed juice was determined by titration with the dye. The effect of storage of gooseberries as such in a refrigerator on the vitamin-C content of the juice was also investigated. The results are shown in Table III. The values per gooseberry given are based on the average of 5 or 6 gooseberries. Table IV shows the effect of storage on the juice itself.

TABLE III.

The effect of storage of gooseberries on the vitamin-C content of the juice.

Period of storage at 0°C. in days.	Average weight of one gooseberry, g.	Average volume of juice obtained from one gooseberry, ml.	Vitamin C per ml. of the juice, mg.	Vitamin C in the total volume of the juice obtained from one gooseberry, mg.
0	11·4	4·7	9·4	44·2
0	11·5	4·5	8·8	39·6
6	9·7	4·0	9·2	36·8
12	8·8	3·2	9·0	28·8
17	8·4	3·0	9·1	27·3
23	7·8	2·8	8·8	24·6

TABLE IV.

The vitamin-C content of the juice during storage at 0°C.

Period of storage in days.	Mg. of vitamin C in 1 ml. of the juice.
0	9·4
3	9·4
6	8·9
12	9·0
26	8·5

For purposes of comparison the vitamin-C potency of gooseberry juice together with that of other fruit juices commonly used in infant feeding is shown in Table V.

TABLE V.

Vitamin-C content of various fruit juices.

Juice.	Mg. of vitamin C in 1 ml. of the juice.
Oranges	0·45 to 0·62
Lemons	0·40 to 0·55
Grape fruits	0·35
Tomatoes	0·22 to 0·31
Indian gooseberries	9·4

The vitamin content per ml. of the juice does not vary on keeping the fruits at 0°C. for 23 days. The total volume and the total vitamin-C content of the juice obtained from one single fruit gradually decrease on storage (Table III). The vitamin-C potency of the juice is not impaired on storage at 0°C. for a period of one month (Table IV). Table V demonstrates the richness of the juice obtained from Indian gooseberries, 1 ml. of the juice being equivalent to about 20 ml. of orange juice, lime juice, or tomato juice.

Other methods of preserving Indian gooseberries.—In addition to drying and preserving the fruits in powder form, which seems very practicable and useful, several other methods of storage were investigated.

Pickling.—The process of pickling, which is much in vogue in Indian homes, is employed for preserving fruits for use when fresh ones are out of season. Various methods are followed with the gooseberry.

(a) *Preservation in concentrated salt solution.*—One of the methods generally employed is to soak the fruits in concentrated salt solution, after keeping them in boiled hot water for about 10 minutes to soften them. It is claimed that the fruits can be preserved in this way for nearly three to four months, provided they are frequently exposed to the sun. Fresh gooseberries were washed well with water and soaked in boiled hot water for about 10 minutes. The excess of water was removed, leaving just enough to immerse them completely. Common salt in the proportion 1 : 4 was added, and after it was completely dissolved, the fruits, together with the salt solution, were stored in a glass vessel. The fruits were occasionally exposed to the sun. The results of vitamin-C analysis of the preserved gooseberries are presented in Table VI. The values per gooseberry are based on the average of 5 to 6 berries.

(b) The other method used is to boil or steam the berries in hot water, and fry them in oil with the final addition of chillie powder and common salt.

This method was tried and found to have destroyed the vitamin to a considerable extent (Table VI). Berries so treated were found to contain after four days' storage about 1 mg. of vitamin C per g. of the pulp, and the total vitamin C in one single berry amounted to 4·5 mg., while the fresh untreated berries contained 6·8 mg. per g. of the pulp, and 57·1 mg. in the whole fruit.

TABLE VI.

Vitamin-C content of gooseberries preserved in various ways.

Material and method of preparation.	Period of storage in days.	Weight of one goose-berry, g.	Weight of fresh pulp from one gooseberry, g.	Vitamin C per g. of the fresh pulp, mg.	Vitamin C contained in the whole fruit, mg.
Fresh fruits (sample A) without treatment.	..	9·5	8·4	6·8	57·1
The same fruits (sample A) preserved by soaking in concentrated salt solution.	37	11·3	10·3	2·8	28·8
The same fruits (sample A) preserved by soaking in concentrated salt solution.	57	11 3	9·9	3·2	31·5
Fresh fruits (sample B) preserved by soaking in concentrated salt solution.	32	8·8	7·6	2·9	22·0
Fresh fruits (sample C) pickled by boiling in water and frying in oil with the addition of chillie powder and salt.	4	6·1	4·6	0·96	4·5

Gooseberries preserved in concentrated salt solution thus retain more vitamin C after storage for about two months than berries preserved by other methods. In this connection the observation of Hoyyaard and Rasmussen (1938), that sodium chloride inhibits the oxidation of vitamin C, is suggestive. Although the salt-preserved gooseberries lost after two months' storage about 50 per cent of their vitamin C reckoned on fresh weight basis, the total quantity of the vitamin present in one fruit amounted to about 28 mg. This amount, according to present estimates. is sufficient to cover human daily requirements.

DISCUSSION.

There is at present little satisfactory information about the prevalence of vitamin-C sub-nutrition in India. But surveys have shown that the consumption of fruits and vegetables by most classes in the population is small and it is possible that the intake of additional vitamin C would result in an improvement

in health. The existence of a cheap and easily available source of the vitamin, as described in the present paper, is therefore of considerable importance.

Indian gooseberries are the richest source of vitamin C among the Indian foodstuffs so far analysed. Damodaran and Sreenivasan (1935) have shown by the usual chemical methods that the berries contain 4·13 mg. of vitamin C per g. of the fresh material. Giri and Doctor (*loc. cit.*) found, by following their modified method of extracting the vitamin in presence of pyrophosphate, that the fresh pulp of the berries contains about 7·2 mg. of vitamin C per g. Later, Spruyt (1938) investigated the vitamin-C content of fruits by the ferment method with the aid of ascorbic-acid oxidase prepared from drumstick, and found that Indian gooseberries contain 5·4 mg. of ascorbic acid per g. fruit. The Indian gooseberry is perhaps the cheapest natural source of vitamin C so far discovered, and from the economic standpoint the wider use of this fruit is feasible and desirable. While the vitamin-C content of fruits and vegetables is generally seriously impaired on drying and storage, gooseberries may retain the vitamin in considerable quantity after treatment in various ways and storage for several months. They may therefore be valuable as a source of the vitamin when fresh berries and many fresh fruits are out of season, or when they are not within the easy reach of the poor. The processes of drying and preservation described above are very easy and simple, and they can be followed in homes for preserving the berries. An aqueous extract of the vitamin can be prepared from the powder, by adding water and pressing out the extract in a cloth or by filtration through a suitable filter. The powder can be used as it is by mixing with other food materials like curd to make it more palatable.

In infant feeding.—Since milk is not a good source of vitamin C, orange juice, lime juice, and tomato juice are often recommended for infants and young children, particularly artificially fed babies. These fruits are sometimes expensive and not available all the year round. Infants are often given one to two spoonfuls (30 ml.) of orange juice daily. This amount of juice provides about 7 mg. to 15 mg. of vitamin C, which is said to be the normal requirement for children per day. The cost of oranges in India is relatively high and Indian gooseberries are a useful cheap alternative. The feeding of infants with about 2 ml. of the juice, which is equivalent to about 30 ml. of orange juice in its vitamin content (18 mg.), may be advocated. It is found that 3 ml. to 4·5 ml. of juice can be obtained from one single fruit and 1 ml. of the juice contains about 9 mg. of vitamin C (Table III). Thus, one gooseberry a day is more than ample for feeding infants with enough vitamin C. The juice can be prepared by straining the pulp in a cloth. It is best given diluted and sweetened with either honey or sugar. The vitamin contained in the juice is quite stable and the juice can be preserved for considerable period without any loss of the vitamin.

SUMMARY.

1. The antiscorbutic potency of Indian gooseberries (*Phyllanthus emblica* Linn.), which chemical investigation had previously shown to be the richest source of vitamin C among Indian foodstuffs, was determined by the biological method based on the weight increase of guinea-pigs, and the reducing substance present in the berries was shown to possess the biological activity of vitamin C.

2. Various methods of preserving the berries in dry powder form and in a fresh state, and the stability of the vitamin under such conditions of preservation, were investigated.

3. The practical use of Indian gooseberries as a dietary source of vitamin C for adults and in infant feeding is discussed.

ACKNOWLEDGMENTS.

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A STUDY OF DIET AND NUTRITION IN NORTH BENGAL, UPPER ASSAM, AND CALCUTTA.

BY

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THE surveys about to be described were carried out on 3 groups : (1) village families in the district of Dinajpore, North Bengal ; (2) families working on tea estates in the Jorehaut district of Upper Assam ; (3) Marwari families in Calcutta. The surveys lasted for a period of approximately three weeks, information about food intake being collected by daily house-to-house visits.

The Dinajpore district is a typical district of North Bengal. The survey in this area was carried out in January–February. The families studied included both villagers who owned and tilled their own lands and also poorer families working on the farms of others as daily labourers in addition to tending their own plots. The latter receive in return for their labour some portion of the food crops or small wages.

The Assam group included labourers of various kinds employed on tea estates receiving weekly wages amounting to Rs. 2 to Rs. 3 per adult male or female and 12 annas to Re. 1 per child. Free huts are provided by the estates and often small plots of cultivable land are available for the labourers. The plots are usually employed for growing vegetables. The diet survey was carried out in March and April. The results of a similar survey previously made in this area have already been published (Wilson and Mitra, 1938).

The survey in Calcutta was carried out in June and was confined to Marwaris. The Marwari community, which is vegetarian by habit, is mainly engaged in business and the professions. The incomes of the families investigated ranged from Rs. 100 to about Rs. 1,000 per month. This group included only 7 families.

Children belonging to the families surveyed and others belonging to families of similar status were weighed and measured and their dynamometer grip readings were taken. The dynamometer figures are those on the graduated scale of the instrument (Down Bros., Ltd., London) and correspond roughly to the weight of as many pounds, e.g., a reading of 10 in the scale can be produced by squeezing the instrument by a weight of 10 lb. The A.C.H. index of nutrition was applied to groups of children aged 7 to 12 in the different areas. The incidence of various diseases and conditions, the majority of which are usually ascribed to dietary

causes, such as angular stomatitis, phrynoderma, gross caries, malocclusion of teeth, enlarged tonsils, xerophthalmia, and Bitot's spots was determined.

Some of the records in the diet survey and the examination of children were found to be unsatisfactory or incomplete and were rejected. In Dinajpore the records relating to the dietary intake of 40 Hindu families were worked up. Satisfactory examination of 1,294 children were carried out ; these belonged to both Hindu and Mohammedan communities distributed over about 35 villages. Similarly, in Jorehaut records of 35 families were analysed and 1,096 children distributed over about half a dozen tea gardens were examined, while at Calcutta satisfactory records of 7 Marwari families were obtained and some 300 Marwari children examined.

The majority of children was both weighed and measured and examined to record the incidence of the conditions previously mentioned. In a small proportion of cases, however, either measurements or examinations were omitted so that the total numbers as stated above do not precisely correspond with those given in the tables.

In working out the results of the diet survey, the usual technique of the Coonoor Laboratories was followed (Aykroyd and Krishnan, 1937*a*). Tables of food values given in Health Bulletin No. 23 (1937) were employed.

ANALYSIS OF DIETS.

Intake per consumption unit of the following was calculated ; total protein, animal protein, total fat, animal fat, carbohydrate, total calories, percentage of calories from cereals, calcium and phosphorus. An attempt was also made to estimate the intake of flavine and vitamin B_6. Table I shows mean intakes of protein, in the three groups of families, with the range of intake in the case of each factor.

Mean calorie intake was fairly high in groups 1 and 3 (the latter consisting of Marwaris of sedentary occupations) ; it was in excess of that recorded by Aykroyd and Krishnan (1937*a*), in the case of South Indian village families (2,400). The mean intake of group 2 was somewhat lower.

Mean protein intake was not far below the standard usually recommended, but the amount of animal protein consumed was small. In the case of the Marwaris in Calcutta, the sources of animal protein were milk or milk preparations, as the Marwaris usually do not take fish, meat, or eggs. Intake of vegetable fat was low and that of animal fat almost negligible in Dinajpore and Jorehaut. The Marwaris, in contrast, use ghee (clarified butter) for cooking purposes and thus consume a good deal of animal fat. Calcium intake was considerably below Sherman's standard in Dinajpore and Jorehaut but reached this standard in the Marwari families.

Intake of iron per consumption unit per day was calculated to be about 25 mg. in Dinajpore, 28 mg. in Jorehaut, and 31 mg. among the Calcutta Marwaris. The figures are based on the data given in Health Bulletin No. 23. It must, however, be remembered that the iron content of foodstuffs is extremely variable, and that the results of the analyses of foodstuffs grown in one part of the country may not be applicable in other areas (Ranganathan, 1938*b*). Calculated on the basis of Ranganathan's (1938*a*) figures, the diet of the Jorehaut (Assam) group was found

TABLE I.

Intake of protein, etc., per consumption unit per day.

	(1) NORTH BENGAL.		(2) ASSAM.		(3) CALCUTTA.	
	Mean intake.	Range.	Mean intake.	Range.	Mean intake.	Range.
Total protein (g.)	71	33 to 98	64	39 to 94	71	62 to 84
Animal protein (g.)	4	..	5	..	10	..
Percentage of animal protein ..	5·4	0·0 to 12·9	7·3	0·0 to 16·7	14·4	5·9 to 28·2
Total fat (g.)	14	6 to 45	13	3 to 33	85	44 to 115
Animal fat (g.)	1	..	1	..	67	..
Percentage of animal fat ..	4·9	0·0 to 16·7	6·5	0·0 to 27·4	78·7	4·4 to 94·3
Carbohydrate (g.)	583	287 to 790	498	307 to 717	392	358 to 458
Total calories	2,740	1,410 to 3,820	2,329	1,429 to 3,434	2,636	2,128 to 3,117
Percentage of calories from cereals.	91·4	85 to 94	86·5	77 to 95	51·3	40 to 62
Calcium (g.)	0·22	0·07 to 1·00	0·27	0·12 to 0·78	0·69	0·45 to 1·14
Phosphorus (g.)	2·24	1·10 to 3·30	1·77	1·05 to 3·36	1·78	1·44 to 2·07

to contain only 7 mg. of available iron. Napier and Das Gupta (1937) observed that out of 100 cases of anæmia in Jorehaut studied by them, practically all were cases of the microcytic-hypochromic, iron-deficiency type.

Intake of phosphorus was high in groups 1 and 2 ; this was due to the high proportion of rice, home-pounded or milled, which the diets contained ; that of calcium, on the other hand, was low. Mean calcium-phosphorus ratios were calculated to be about 1 : 10 (Dinajpore) and 1 : 7 (Jorehaut). Among the Marwaris at Calcutta, however, intakes of calcium and phosphorus were both ' up to standard ', with a calcium : phosphorus ratio 1 : 3, owing to the habit of taking milk and atta (whole wheat).

The diets of groups 1 and 2 contained only very small quantities of vitamin A. It was calculated that the amount of carotene consumed would on the average supply less than 500 International Units of vitamin A per day in group 1 and about 1,500 I.U. in group 2. The diets of group 3, however, supplied daily about 500 I.U. of vitamin A, and sufficient carotene to furnish about 4,000 I.U. in addition. Vitamin-B_1 intake in all three groups was probably adequate. The Bengal group consumed only home-pounded parboiled rice as the chief staple, while the Assam group consumed both milled and home-pounded parboiled rice. The staple foodstuff of the Marwaris was atta (whole wheat), consumed in the form of brown bread. Vitamin-B_1 intake was calculated to be about 800 I.U. per consumption unit per day in group 1, about 700 I.U. in group 2, and about 1,000 I.U. in group 3.

Using the results of Wilson and Roy (1938) an attempt was made to estimate roughly intake of flavine and vitamin B_6. Mean intake of the former was estimated to be about 1·3 mg., 1·1 mg., and 0·7 mg. and that of the latter about 400, 300, and 300 rat units in the three groups, respectively.

The composition of the diet as regards foodstuffs was very similar in Dinajpore and Jorehaut. The families surveyed in these areas consumed similar meals consisting of home-pounded or milled rice, dhal, vegetables, and small fish. A few consumed milk and goat's meat. In Jorehaut, the Dóms (a sub-caste of depressed class Hindus) consumed fresh beef occasionally, while dry fish and dry beef were also included in the diet in small quantities. Average intake of the more important foodstuffs per consumption unit per day was as follows :—

•	Group 1 (Dinajpore), oz.	Group 2 (Jorehaut), oz.
Parboiled rice ..	25·0 (home-pounded)	19 4 (mostly milled)
Pulses	0·4	0·9
Non-leafy vegetables ..	7·0	4·4
Leafy vegetables ..	0·2	0·8
Fish, eggs, and meat ..	0·7	0·7
Mustard oil ..	0·3	0·3

In Dinajpore, rice was generally pounded in small quantities at a time, to last for 3 or 4 days, from the paddy stored in the house and no milled rice was used. The commonest varieties of pulses used were lentils, green gram (*Phaseolus radiatus*), and black gram (*Phaseolus mungo*). Of the non-leafy vegetables, brinjal (*Solanum melongena*) and potato were commonly eaten ; other vegetables were pumpkin (*Cucurbita maxima*), calabash cucumber (*Lagenaria vulgaris*), and green plantains. The amount of leafy vegetables consumed was very small. Mustard oil was extracted from the seeds by the old-type village wooden mills and appeared to be pure.

In Jorehaut, the rice was parboiled and mostly milled ; very little home-pounded rice was used. Lentils and red gram (*Cajanus indicus*) were the pulses mainly taken. Of the non-leafy vegetables, brinjal and potato were the usual varieties, as in Dinajpore ; other vegetables were pumpkin, calabash cucumber, green plantains, bitter gourd (*Momordica charantia*), cauliflower, tamarind, and tomatoes. Amaranth (*Amaranthus gangeticus*) and drumstick (*Moringa oleifera*), with small amounts of cabbage, were the usual leafy vegetables. Some of the workers were accustomed to spend a portion of their earnings on crude alcoholic drinks.

In group 3, the Marwaris in Calcutta, average intake of the chief foodstuffs per consumption unit per day was as follows :—

	Oz.
Rice	3·0
Atta (brown bread)	10·3
Pulses	2·4
Non-leafy vegetables	6·2
Leafy vegetables	0·1
Milk and milk preparations	11·7
Ghee (clarified butter)	2·0
Fruits	3·1
Sugar	0·6

Brown bread was the usual staple diet. A small quantity of parboiled or raw, rather highly milled, rice was also taken. The usual pulses used were Bengal gram (*Cicer arietinum*), red gram, green gram, black gram (in the form of papads), and occasionally peas. Lentils, commonly consumed in Dinajpore or Jorehaut, were not seen in any Marwari house. Many varieties of locally available non-leafy vegetables were consumed. In addition, it is the custom among Marwari families in Calcutta to import some dried vegetables, resembling French beans, from their native districts in Rajputana. Leafy vegetables were not much consumed ; only small amounts of amaranth leaves and mint were included in the diet. Milk and curds, with some sugar, usually formed a part of each meal and the intake of milk and milk products was fairly high. Fruits included mango, banana, pine-apple, apple, grapes, peaches, tree tomatoes (*Cyphomandra betacea*), water melon, guavas, oranges, etc.

Survey of school children.

The height, weight and dynamometer grip of the children examined in the different areas, and the incidence of various clinical conditions observed among

them, are shown in Tables II to V. In recording the presence of caries, probe and mirror were not used and only caries which could easily be seen on rapid inspection was noted. 'Malocclusion' of teeth in the present investigation, means one or more of the following conditions (illustrated in Plate XVIII, figs. 1, 2, and 3) in the upper or lower jaw (Nicholls. 1936; Wilson and Mitra, *loc. cit.*): (*a*) the teeth were grossly rotated about their axes (Fig. 1); (*b*) the teeth were unduly pushed forward with overlapping of crowns, due to overcrowding, the lateral incisors having erupted behind the central incisors in many instances (Fig. 2); (*c*) gaps were present between incisors (Fig. 3). Enlargement of tonsils was recorded only when such enlargement was visible on asking the child to open the mouth widely and protrude the tongue without using a tongue depressor.

The frequency of gross caries was very constant throughout the groups, being in the neighbourhood of 20 per cent. Malocclusion of teeth was more frequent in the Calcutta group. In groups 1 and 2 enlarged tonsils appeared to be somewhat more common among girls. The Calcutta boys showed a higher incidence than boys in rural Bengal and Assam.

The incidence of xerophthalmia and Bitot's spots in Dinajpore was smaller than that observed in Jorehaut and in a previous survey in Assam (Wilson and Mitra, *loc. cit.*). Fewer cases were recorded among the girls than among the boys. A fair number of cases of angular stomatitis was, however, encountered in Dinajpore. The incidence of phrynoderma in Assam and Dinajpore was lower than that recorded in South Indian schools and hostels (Aykroyd and Rajagopal. 1936; Aykroyd and Krishnan, 1937*b*) though the skin was dry and rough in many cases. No definite case of angular stomatitis, phrynoderma or Bitot's spots was seen among the Marwaris; this was in agreement with the results of previous surveys in Calcutta (Wilson, Ahmad and Mitra, 1937; Wilson and Mitra, *loc. cit.*).

It is interesting to observe that the boys in group 1 (Dinajpore) had a definite advantage as regards average weight over those in group 2 (Assam): the weights of the former corresponded closely with those of the comparatively well-nourished group of Marwari boys in Calcutta. The weight averages of children belonging to the same age groups, obtained in a previous investigation (Wilson and Mitra, *loc. cit.*) were of a similar order. Similarly, the girls in group 1 were on the average superior in weight to those in group 2. The boys in group 1 had higher average weights than those recorded in various groups measured in South India, which included children of the poorer class in three towns (Aykroyd and Rajagopal, *loc. cit.*) and a group of village children in the Mayanur district of the Madras Presidency (Aykroyd and Krishnan, 1937*b*). The recorded age of children of the poorer classes in India is often inaccurate. but this probably does not greatly influence the trend of mean weight and height averages in various groups, since errors tend to cancel each other out.

As regards average height, the boys in rural Bengal and the Calcutta group had an appreciable advantage over the children in the Assam group.

The comparatively good physical development of children in the districts of rural Bengal in which surveys have been made may perhaps be ascribed to the fact that in ordinary circumstances the main ingredient in the diet is freshly prepared home-pounded parboiled rice, consumed in fairly abundant quantities.

PLATE XVIII.

Malocclusion of teeth.

Fig. 1.—Rotation of upper right lateral incisor.
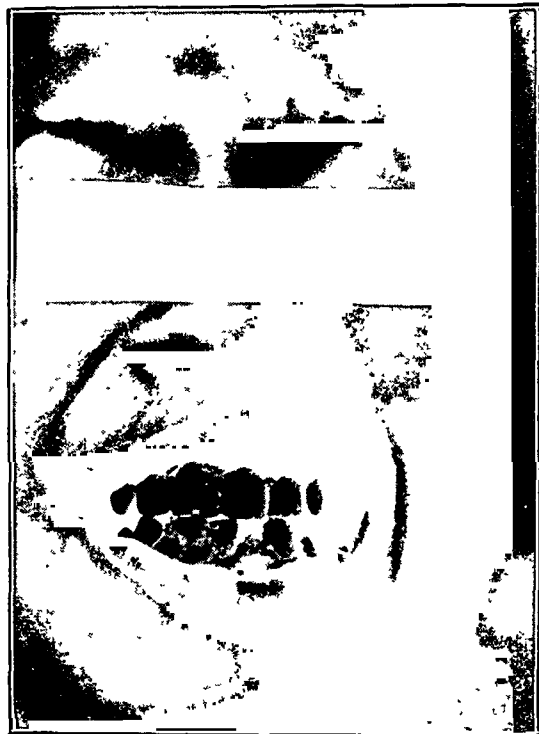
Fig. 2.—Lower left lateral incisor erupting behind central incisors.
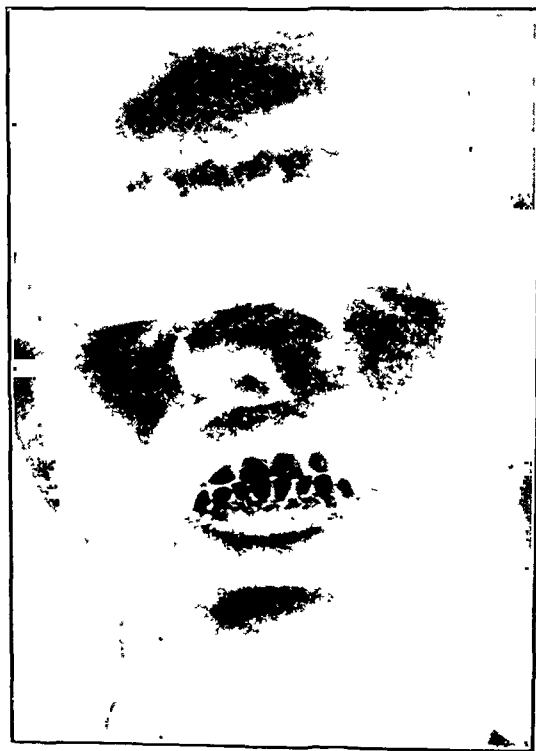
Fig. 3.—Overcrowding of lower incisors and gaps between upper lateral incisors and central incisors.

TABLE II.

Height, weight, and dynamometer grip of children in Dinajpore, Northern Bengal (group 1).

Age group.	NUMBER WEIGHED AND MEASURED.				HEIGHT (INCHES).				WEIGHT (LB.).				DYNAMOMETER GRIP.			
	HINDU.		MOHAMMEDAN.		HINDU.		MOHAMMEDAN.		HINDU.		MOHAMMEDAN.		HINDU.		MOHAMMEDAN.	
	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.
4	10	5	15	7	35·6	35·7	37·3	34·5	26·9	27·6	28·0	24·2	4·7	4·4	6·1	5·5
5	35	17	38	21	39·8	37·1	39·7	38·2	33·0	29·2	31·2	29·2	7·8	5·8	7·7	5·9
6	41	15	39	9	41·3	39·5	42·2	41·1	35·1	32·2	34·7	33·2	9·2	8·3	10·7	7·7
7	85	45	74	38	45·0	42·5	45·0	42·7	41·2	36·8	40·0	36·1	13·3	10·2	13·6	10·4
8	63	20	63	22	47·6	45·2	47·8	46·0	48·1	42·8	45·9	41·6	17·8	14·8	17·0	13·2
9	70	12	41	10	49·6	47·4	49·5	47·2	51·1	45·8	49·5	46·0	19·9	15·8	19·1	14·9
10	34	9	35	13	50·9	48·5	49·9	47·9	54·8	50·8	52·6	46·1	21·8	17·7	20·0	16·5
11	46	3	42	7	51·4	50·7	51·8	50·1	59·5	53·7	55·7	50·6	24·8	18·7	21·7	19·7
12	92	9	88	15	54·0	53·3	53·9	50·2	64·2	57·3	60·9	53·4	25·8	24·1	25·1	19·7

TABLE III.

Height, weight, and dynamometer grip of children in Jorehaut, Assam (group 2).

Age group.	NUMBER WEIGHED AND MEASURED.		HEIGHT (INCHES).		WEIGHT (LB.).		DYNAMOMETER GRIP.	
	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.
5	15	6	34·6	34·9	24·4	25·3	4·5	4·0
6	41	29	38·4	37·5	30·1	28·4	7·7	5·3
7	85	60	41·8	41·3	34·5	33·1	10·3	8·5
8	74	48	44·3	43·7	39·1	37·6	13·4	11·4
9	48	35	46·1	44·9	41·6	40·4	15·0	14·0
10	42	27	46·8	46·7	42·9	42·5	17·4	14·5
11	63	51	48·1	46·7	46·6	43·8	18·7	16·0
12	179	129	51·3	51·3	53·8	53·7	22·6	20·5
13	28	10	54·2	52·6	62·5	56·7	28·2	22·0
14	23	13	55·8	53·9	66·2	64·3	30·9	27·8
15	16	10	57·4	55·1	75·0	66·4	34·1	27·1
16	25	11	58·0	56·0	78·0	69·3	36·8	27·0

TABLE IV.

Height. weight, and dynamometer grip of Marwari boys in Calcutta (group 3).

Age group.	Number weighed and measured.	Height (inches).	Weight (lb.).	Dynamometer grip.
7	14	44·7	41·6	22·7
8	17	46·5	45·2	24·2
9	14	49·0	50·2	23·0
10	48	50·9	54·9	24·6
11	69	53·3	62·1	25·3
12	79	53·8	64·3	25·7
13	36	57·2	75·7	27·4
14	22	58·9	85·9	28·4

TABLE V.

Incidence of various clinical conditions observed in North Bengal (1), Assam (2), and Calcutta (3).

1. NORTH BENGAL.

Community.	Sex.	Total number examined.	Angular stomatitis.	Phryno-derma.	Xeroph-thalmia.	Bitot's spots.	Gross caries.	Malocclusion of teeth.	Enlarged tonsils.
Hindu ⟨ ..	Boys	539	34 (6·3 per cent.)	3 (0·5 per cent.)	11 (2·0 per cent.)	5 (1·0 per cent.)	114 (21·1 per cent.)	48 (8·5 per cent.)	61 (11·3 per cent.)
	Girls	135	9 (6·7 per cent.)	1 (0·7 per cent.)	1 (0·7 per cent.)	0	27 (20·0 per cent.)	13 (9·6 per cent.)	29 (21·5 per cent.)
Mohammedan ⟨ ..	Boys	425	42 (10·0 per cent.)	7 (1·6 per cent.)	9 (2·1 per cent.)	3 (0·7 per cent.)	87 (20·5 per cent.)	40 (9·4 per cent.)	57 (13·4 per cent.)
	Girls	125	12 (9·6 per cent.)	0	1 (0·8 per cent.)	0	27 (21·6 per cent.)	13 (10·4 per cent.)	33 (26·4 per cent.)

2. ASSAM.

Community.	Sex.	Total number examined.	Angular stomatitis.	Phryno-derma.	Xeroph-thalmia.	Bitot's spots.	Gross caries.	Malocclusion of teeth.	Enlarged tonsils.
Mostly Hindu ⟨ ..	Boys	670	7 (1·0 per cent.)	9 (1·2 per cent.)	0	14 (2·1 per cent.)	135 (20·0 per cent.)	72 (10·7 per cent.)	66 (9·8 per cent.)
	Girls	426	2 (0·5 per cent.)	1 (0·2 per cent.)	0	5 (1·2 per cent.)	79 (18·5 per cent.)	51 (12·0 per cent.)	82 (19·2 per cent.)

3. CALCUTTA.

Community.	Sex.	Total number examined.	Angular stomatitis.	Phryno-derma.	Xeroph-thalmia.	Bitot's spots.	Gross caries.	Malocclusion of teeth.	Enlarged tonsils.
Marwari ..	Boys	241	No cases	No cases	No cases	No cases	53 (21·9 per cent.)	92 (25·7 per cent.)	62 (25·7 per cent.)

The A.C.H. index.—Selection by the index was found to be high in groups 1 and 2, in which the percentages selected were 42·4 and 53·4 respectively. These are higher percentages than any previously recorded in investigations in India. In the Calcutta group the percentage was only 4·1 per cent.

A number of emaciated children, 71 boys and 18 girls, were selected in Dinajpore, and their A.C.H. indices determined; it was found that of these 39 per cent in the case of the boys and 44 per cent in the case of the girls were not selected, while, on the other hand, among the children selected by the index there were some who appeared to be healthy and of good physique. It seems questionable whether the A.C.H. index can be applied with success to Bengalee children, though Aykroyd, Madhava and Rajagopal (1938) found it a useful supplementary method of assessing state of nutrition in South India. The fact that it failed to select a higher percentage of emaciated children does not, however, altogether invalidate its use since it was never intended by its originators to be applied to such children.

SUMMARY.

1. A diet survey has been carried out of 40 agricultural families in the district of Dinajpore (Bengal), 35 labouring class families in the district of Jorehaut (Assam), and 7 well-to-do Marwari families in Calcutta.

2. The height and weight measurements of about one thousand children belonging to these families and others of similar economic status in each of the three different areas were taken. Records were obtained of dynamometer grip and of the incidence of angular stomatitis, phrynoderma, xerophthalmia, etc. The A.C.H. index was applied to children in the age group 7 to 12.

3. On the whole, the state of nutrition of the children in Dinajpore and at Calcutta was fairly satisfactory. The lower height and weight averages in the various age groups among the Jorehaut children, as compared with those determined in the other groups, probably indicate a poor state of nutrition.

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A STUDY OF DIET AND NUTRITION IN ORISSA.

BY

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THE diet surveys described in this paper were carried out in the Puri district of Orissa Province in the months of January and February - 1938. The surveys lasted from 7 to 10 days and the total number of families investigated was 33, comprising 163 individuals. These have been divided into the following three groups :—

I. A relatively well-to-do urban group of domiciled Bengalees.

II. Labourers, fishermen, etc., living in Puri town.

III. A rural group including :—

    (*a*) families free from leprosy, and

    (*b*) families in the same area containing one or more members suffering from leprosy, and at the time of the investigation under treatment for the disease at local hospitals and dispensaries.

The diets consumed by the various groups are described in terms of foodstuffs in Table I and in terms of food factors in Table II. The usual technique was followed (Aykroyd and Krishnan, 1937*a*). Table I also includes the results of a survey in the Cuttack district of Orissa carried out in collaboration with Dr. D. C. Wilson, the full report of which has not yet been published.

The diets included very small amounts of various condiments. In group I the consumption of sugar was about 0·6 oz. per consumption unit per day; intake of sugar in the other groups was negligible. Only a few families in the entire group consumed fruit in small quantities once or twice during the period of investigation.

TABLE I.

Principal foodstuffs consumed by the various groups (oz. per consumption unit per day).

Foodstuffs.	Cuttack.	Group I (Puri).	Group II (Puri).	Group III (a) (Puri).	Group III (b) (Puri).
Total cereals ..	19·4	18·0	17·0	24·6 (one family used atta).	21·1
Atta (whole wheat flour)	0·6	2·0	..	..	..
Rice	18·9 (parboiled undermilled).	16·0 (largely parboiled home-pounded).	17·0 (largely parboiled milled).	24·6 (parboiled home-pounded).	21·1 (parboiled home-pounded and milled).
Pulses	1·0	1·5	0·9	0·9	0·6
Roots and tubers ..	3·3	2·9	0·3	1·6	0·9
Leafy (green) vegetables	1·4	2·0	0·9	0·3	0·4
Other vegetables ..	2·5	5·8	1·6	6·1	5·1
Milk and milk products	0·3	6·5	..	(one family used milk).	..
Fish and meat ..	0·6	1·5	1·4	0·6	0·1
Clarified butter (ghee)	..	0·2	..	..	..
Vegetable oil ..	0·3	0·6	0·2	0·1	0·1
Nuts and oil seeds ..	..	0·1	0·2	0·1	..

ANALYSIS OF THE DIET.

Mean calorie intake was fairly adequate in group I and in group III (a) though the figures are lower than those recorded in the case of village families of Delhi Province (Shourie, 1939). In groups II and III (b) caloric intake was low. The

TABLE II.

Intake of calories, etc., in the various groups (per consumption unit per day).

Foodstuffs.	Group I.		Group II.		Group III (a).		Group III (b).	
	Mean.	Range.	Mean.	Range.	Mean.	Range.	Mean.	Range.
Calories	2,506	1,850—2,947	1,953	1,476—2,844	2,742	1,894—3,925	2,282	1,732—3,207
Calories from cereals	1,785	..	1,675	..	2,449	..	2,099	..
Percentage	71·3	..	85·8	..	89·3	..	92·1	..
Total protein	78·0	119·3—52·3	52·6	35·8—80·0	75·8	55·5—119·4	59·1	44·9—82·2
Animal protein	15·0	8·5—25·0	8·9	1·6—26·5	5·4	3·2—16·0	0·5	0·0—3·4
Percentage	19·2	..	16·9	..	7·1	..	1·0	..
Total fat	35·7	22·8—51·6	12·7	6·2—25·6	8·8	6·7—17·0	7·6	5·4—12·2
Animal fat	12·6	2·1—25·3	0·8	0·3—1·9	0·9	0·8—2·3	..	..
Percentage	32·2	..	6·2	..	9·7	..	0·5	..
Carbohydrates	476·2	350·1—555·1	413·1	315·5—600·3	583·8	408·3—815·7	479·7	367·9—681·0
Total calcium	0·64	0·39—1·09	0·28	0·14—0·71	0·28	0·13—0·70	0·21	0·12—0·45
Total phosphorus	1·88	1·09—2·42	1·35	0·80—2·28	2·31	1·60—3·48	1·87	1·48—2·54
Total iron	28·7	19·8—40·0	23·4	12·9—43·8	28·6	17·0—49·6	24·0	17·6—38·6
Carotene	2,284	649—6,123	1,141	227—3,906	826	304—2,499	692	182—1,951

mean protein intake in all groups was not much below the usual standards recommended. The diets of groups I, II, and III (*a*) contained animal proteins derived from milk and fish in group I, and mostly from fish in groups II and III (*a*). Group III (*b*) consumed almost negligible amounts of animal protein; one family only consumed a small amount of fish on two occasions. The intake of animal protein in all groups was less than the intake usually recommended, and there was graded drop from group I to group III (*b*).

Intake of total fat was low in all the groups and there was a gradual fall from group I to group III (*b*). Animal fat in groups II, III (*a*), and III (*b*) was negligible and was the least in group III (*b*). Clarified butter (ghee) was used to a very small extent by group I. Calcium intake was up to Sherman's standard in group I, but below in the other groups. The phosphorus content of the diet was high, due to the high consumption of parboiled rice—home-pounded or milled. The calcium : phosphorus ratio of these groups, as worked out from the mean figures, is as follows :—

Group I	..	..	..	.. 1 to 3
Group II	..	..	..	.. 1 to 5
Group III (*a*) ..	..	..	.. 1 to 8
Group III (*b*) ..	..	..	.. 1 to 9

Iron intake per unit per day varied from a mean of 28·7 for group I, 23·4 for group II, and 28·0 and 24·0 for groups III (*a*) and III (*b*), respectively. Carotene and vitamin-A content was maximum in group I and least in group III (*b*).

The majority of families consumed either home-pounded parboiled or parboiled rice machine-milled to a moderate degree. There appears to be little beri-beri in these districts. The most important pulse in the diets was green gram (*Phaseolus radiatus*). Dhal arhar (*Cajanus indicus*) was also commonly consumed, though in smaller quantities.

The most important leafy vegetable was amaranth (*Amaranthus gangeticus*) and the most important non-leafy vegetable brinjal (*Solanum melongena*). A greater variety of vegetables was consumed by the Cuttack group and the domiciled Bengalees (group I). Cabbages, cauliflowers, and tomatoes were sometimes taken by these groups during the period of survey.

Intake of meat was almost negligible, but fish in small quantities was consumed by the majority of families in most of the groups. Except in the case of group I, milk and milk products were for practical purposes absent from the diet. As regards vegetable fats, the oil most commonly used in this part of India is rape oil; this was consumed in very small quantities.

DIET AND LEPROSY.

According to the Report of the Public Health Commissioner with the Government of India (1930), a leprosy survey carried out in nine divisions of the Puri district revealed 3,825 cases. In the Public Health Commissioner's Report for 1931 and 1936 Puri district is included among the regions in which the incidence of leprosy is high.

The Public Health Commissioner (1930) suggests that the distribution of leprosy is influenced by the following factors :—

(*a*) The soil. When the soil is poor and vegetable and fodder crops do not thrive, and cereal crops are apt to fail, a high incidence of leprosy occurs.

(*b*) Leprosy is common in areas in which vegetables and milk products are not consumed in adequate quantities.

The above conditions are present in most parts of Orissa.

McCarrison (1936) in his Cantor Lectures pointed out that an association may exist between leprosy and malnutrition. Aykroyd and Krishnan (1939) carried out a diet survey of 14 families in a suburb of Madras, 13 of which contained cases of leprosy; they found that the diet consumed was deficient in quantity and quality.

The present survey provides some support for the hypothesis that malnutrition is a factor influencing the susceptibility of populations to leprosy. The families containing cases of leprosy included in the present survey consumed a deficient diet and were somewhat worse off in this respect than the other groups. It is noteworthy that in the leprosy group the percentage of total calories obtained from rice was the highest.

State of Nutrition.

A survey of children was carried out in the districts of Cuttack and Puri in Orissa Province. The methods of Aykroyd and Rajagopal (1936) in South India were followed. In both places rural and urban children were examined for height, weight, A.C.H. measurements, and conditions associated with deficient intake of protective foods, i.e., phrynoderma, stomatitis, and Bitot's spots. The incidence of gross dental caries and malocclusion of teeth was also studied.

In the Cuttack district 561 children aged 5 to 14 (470 boys and 90 girls) were examined; children of all classes were included, but the great majority belonged to poorer class families consuming a diet resembling that shown in Table I. In the Puri district the examinations covered 837 children of the same age groups, 697 boys and 140 girls. The social status of the children was much the same as that of the children in the Cuttack group. Of the 837 children 402 were resident in Puri town and 435 in the rural area of Khurda; the diets consumed by the majority of these children corresponded roughly to those of groups II and III (*a*) respectively. For recording weight a spring balance was used, the accuracy of which was tested from time to time by placing known weights on the balance. Children were weighed with shirts and dhoties without shoes. Height was recorded without shoes. Ages were obtained from the school records; their accuracy cannot be guaranteed since they were based on estimates made by the teachers on the child's admission to school.

Table III gives the mean heights and weights of boys in the Cuttack and Puri districts. Averages of groups of boys in Delhi Province in North India and in South India are shown for the sake of comparison. In Charts 1 and 2 these figures are shown graphically.

TABLE III.

Average height and weight for age of boys in Orissa (Cuttack and Puri) and in Delhi Province and South India.

Age.	CUTTACK.			PURI.			NAJAGARH (DELHI PROVINCE).*			SOUTH INDIA.†		
	Number.	Mean height.	Mean weight.	Number.	Mean height.	Mean weight.	Number.	Mean height.	Mean weight.	Number.	Mean height.	Mean weight.
5	23	40·6	33·1	40	40·3	32·6	14	42·5	37·8	..	..	..
6	28	43·8	37·4	65	43·2	37·0	72	43·6	39·0	122	42·8	36·6
7	40	45·2	40·2	110	45·6	41·3	142	45·3	42·2	184	44·8	39·2
8	63	46·9	44·1	130	46·3	44·9	170	47·3	45·8	186	46·4	42·1
9	83	49·6	50·2	97	49·5	49·5	183	48·7	48·8	177	48·0	46·5
10	83	50·9	55·2	96	51·6	54·3	164	49·9	52·0	203	50·4	50·2
11	43	53·2	59·0	46	52·9	58·6	135	51·2	55·8	168	51·4	51·6
12	38	51·6	63·8	52	54·7	63·7	119	53·5	61·1	133	52·5	55·6
13	21	55·4	65·5	29	56·6	70·7	106	55·4	66·6	107	55·9	64·5
14	48	59·9	86·6	32	59·2	85·5	108	57·7	73·6	53	56·6	68·7
15	..	..	..	..	..	..	73	60·9	84·5	49	58·5	74·1
16	..	..	..	..	..	..	38	61·8	91·0	..	..	..
17	..	..	..	..	..	..	15	64·4	105·1	..	..	..

* Shourie (1939).   † Nutrition Research Laboratories.

CHART 1.

Height-age averages of boys in Orissa and other parts of India.

CHART 2.

Weight-age averages of boys in Orissa and other parts of India.
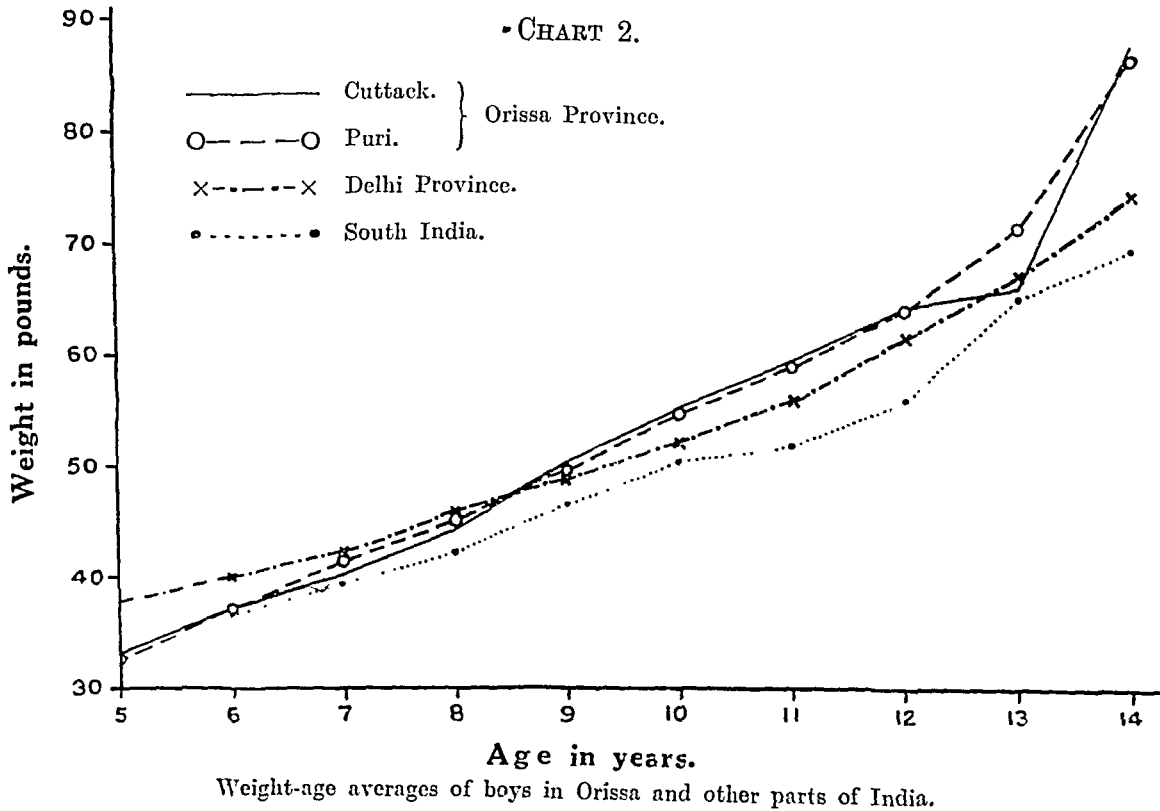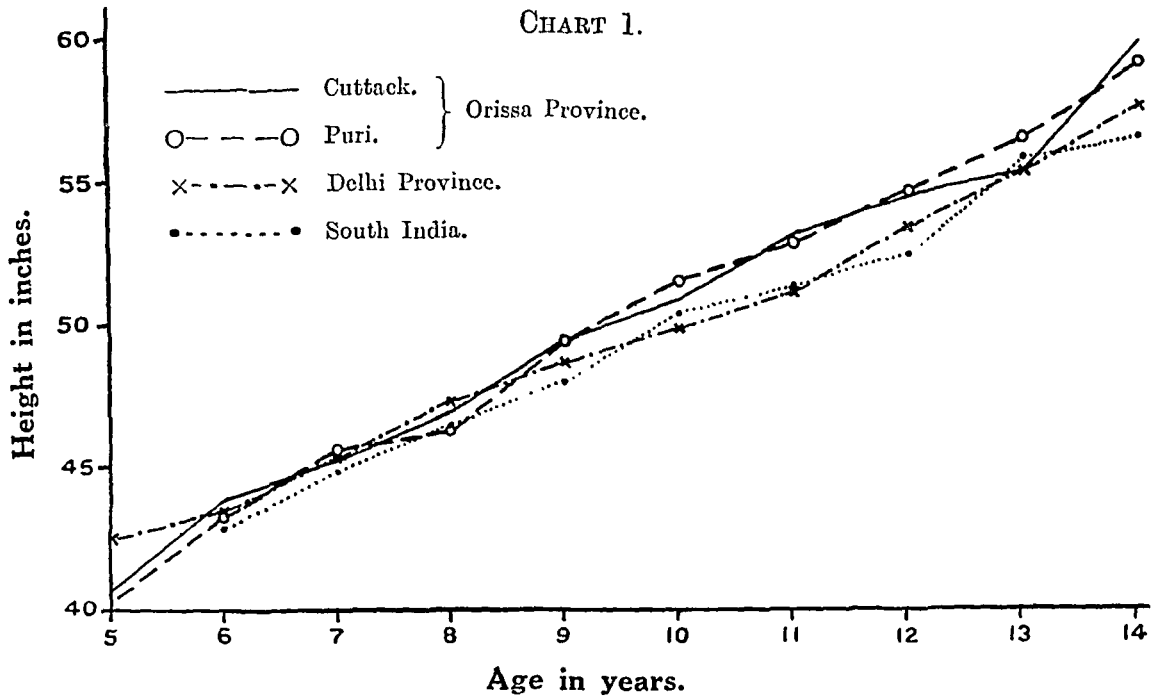
Table IV shows the incidence of phrynoderma, stomatitis, and Bitot's spots in children in the various areas. The incidence was remarkably high. In Table V the relation between the presence of deficiency disease and selection by the A.C.H. index is shown, while Table VI gives the incidence of gross dental caries and malocclusion of teeth respectively.

TABLE IV.

The incidence of certain deficiency diseases in the various groups.

Area.	Number examined.	Number showing signs.	Percentage of those examined.	Number with phrynoderma.	Percentage of those examined.	Number with stomatitis.	Percentage of those examined.	Number with Bitot's spots.	Percentage of those examined.
Cuttack ..	561	209	37·2	145	25·8	92	16·4	29	5·2
Puri town ..	402	161	40·0	88	21·9	91	22·6	26	6·5
Khurda sub-division.	434	187	43·1	106	24·4	135	31·2	26	6·0

TABLE V.

The relation between deficiency diseases and selection by the A.C.H. index
(boys between 7 and 12).

Area.	Number examined for A.C.H.	Number selected.	Percentage.	Number showing one or more signs.	Number selected with signs.	Percentage.	Number not showing signs.	Number selected without signs.	Percentage.
Cuttack ..	349	136	36·0	138	69	50	207	67	32·0
Puri town ..	280	99	35·0	114	53	47	166	46	22·0
Khurda sub-division.	251	75	29·9	139	52	37	112	23	21·0
TOTALS ..	880	300	34·0	391	174	45	485	136	28·0

TABLE VI.

The incidence of gross dental caries and malocclusion.

		CUTTACK.			PURI TOWN.			KHURDA SUB-DIVISION.		
		Number examined.	Number showing gross caries.	Percentage.	Number examined.	Number showing gross caries.	Percentage.	Number examined.	Number showing gross caries.	Percentage.
Caries.	Boys	470	179	38·1	346	120	34·7	345	147	42·6
	Girls	90	33	35·0	50	16	32·2	90	34	37·7
Malocclusion.			Number showing malocclusion.			Number showing malocclusion.			Number showing malocclusion.	
	Boys 9 to 14 only.	316	181	57·2	216	149	68·9	136	94	69·1

In examining the children for dental decay, probe and mirror were not used so that the figures represent only the incidence of gross caries. 'Malocclusion' in the present connection means that the children showed one or more of the following conditions :—

Upper jaw :—

(a) Teeth crowded with overlapping of crowns.
(b) Teeth rotated about axes.
(c) Large gap between incisors (central or lateral).
(d) Incisor protruding in the shape of a fan.
(e) Lateral incisors erupted behind central incisors.
(f) Other irregularities.

Lower jaw :—

(g) Teeth crowded with overlapping of crowns.
(h) Rotation about axes.
(i) Lateral incisors eruption behind central incisors.
(j) Other irregularities.

DISCUSSION.

The percentage incidence of stomatitis, Bitot's spots, and phrynoderma was high in the children examined, and this is an indication of the prevalence of malnutrition. The incidence is higher than that recorded by Aykroyd and Krishnan (1937b) in village children in the Madras Presidency and by Mitra (1939) in rural Bengal and Assam. The type of diet consumed in Orissa, as revealed by the present investigation, is not very different from that of families in Madras, Bengal, and Assam studied in the surveys mentioned. It is therefore somewhat difficult to explain the higher incidence of deficiency disease in the Orissa children. The difference, however, is only one of degree; the conditions mentioned were observed in the surveys carried out by Aykroyd and Krishnan (1937b) and Mitra (loc. cit.). It appears that in many parts of India the incidence of stomatitis and phrynoderma varies with season, a fact which is presumably related to seasonal changes in diet. Possibly the investigation in Orissa was carried out at the season of greatest prevalence.

The survey shows that malnutrition is extremely prevalent and severe in this part of India. It may be mentioned that ankylostomiasis and malaria are also very prevalent in Orissa. Many of the children examined had enlarged spleens; the high incidence of ankylostomiasis was confirmed by jail authorities. There may be a relation between the frequency of deficiency disease in school children and the high incidence of malaria and hookworm.

Aykroyd, Madhava and Rajagopal (1938) observed a close relation between the presence of deficiency disease and selection by the A.C.H. index. In the Orissa children there was some correlation, since there was a higher percentage of selected cases among children with deficiency diseases than among children free from such diseases. Nevertheless, the fact that a large proportion of children showing signs were not selected, and vice versa, suggests that the index cannot be recommended for practical use in this part of India. It is perhaps scarcely to be expected that any single morphological index would be suitable for application throughout a country in which there is such diversity of race and body build.

Average height in the various age groups of boys in Orissa was found to be very similar to that of boys in other parts of India. As regards weight, they were on the average heavier at all ages than the South Indian children weighed in previous investigations carried out by the Laboratories. The North Indian groups, investigated by Shourie (loc. cit.), consumed a diet based on wheat and containing milk in reasonable quantities which appears to be of considerably higher nutritive value than the Orissa diets. The diets of the Madrassi children, the majority of whom were rice-eaters, closely resembled the diets consumed in Orissa. Further accumulation of data is necessary to throw light on the relation between the height and weight of Indian children, diet, and other factors including race.

SUMMARY.

1. The diets of groups of families living in the Cuttack and Puri districts of Orissa Province have been studied. One of the groups consisted of families containing cases of leprosy.

2. The diets of the majority of families consisted mainly of parboiled rice with vegetables and fish and vegetable oil in small quantities. Intake of milk and milk products in all the groups except one was negligible. The diet of the families with cases of leprosy was particularly deficient in protective foods.

3. The incidence of signs of deficiency disease: stomatitis, xerophthalmia, and phrynoderma, was high in children attending schools in the districts concerned.

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OBSERVATIONS ON THE VITAMIN-B₁ EXCRETION OF FOUR HEALTHY SUBJECTS LIVING ON DIFFERENT INDIAN DIETS.

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HARRIS and his colleagues (Harris and Leong, 1936; Harris, Leong and Ungley, 1938) have published extensive data on the vitamin-B₁ excretion of individuals under different conditions. They have shown that the daily output of this vitamin varies with the dietary intake and the response to a test dose is graded according to the past dietary history of the individual. These authors concluded that the determination of the level of excretion of this vitamin in the urine together with the study of the degree of response to a test dose furnishes a reliable estimate of the state of vitamin-B₁ nutrition of the individual. In order to find out if common Indian diets are able to supply the vitamin-B₁ requirements adequately, we have investigated the vitamin-B₁ excretion of four healthy subjects living on typical Indian diets. The special interest of the investigation lay in the fact that these subjects belonged to different parts of India and lived on diets typical of those areas. In addition to their normal diets the effect of certain modifications to the diets was also studied.

The following is a brief description of the average normal diets of the four subjects :—

(*i*) B. A., Punjab.

Wheat atta as chapati 4 oz.; mutton, curried with vegetables 4 oz.; toasted brown bread 3 oz.; eggs, fried or in puddings 3 oz.; milk as such, curd or curd shake 8 oz.; butter 1 oz.; ghee 2 oz.; vegetables in curries 8 oz.; fruit 6 oz.; sugar as sweets, jams or puddings 2 oz.; tea, etc.

This diet contains 1,425 µg. vitamin B₁ (475 International Units) and 2,300 calories.

(*ii*) R. C. G., East Bengal.

Rice half-milled, boiled 5 oz.; rice fried 1 oz.; white bread 2 oz.; milk 8 oz.; lentils, as curry with boiled rice 2 oz.; fish, fried or curried 4 oz.; vegetables in curries 6 oz.; mustard oil in vegetable or fish curries 2 oz.; butter ½ oz.; sugar 2 oz.; tea, etc.

This diet furnishes 1,350 µg. of vitamin B₁ (450 International Units) and 2,250 calories.

(*iii*) G. R., West Bengal.

> Rice half-milled, boiled 4 oz.; white bread 2 oz.; wheat atta as chapati 2 oz.; wheat flour as luchi 2 oz.; eggs 2 oz.; fish, fried or curried 4 oz.; dhal 2 oz.; vegetables in curries 4 oz.; fruits 2 oz.; sugar as sweets 2 oz.; mustard oil 2 oz.; chutnis; tea, etc.
>
> This diet furnishes 1,275 μg. of vitamin B₁ (425 International Units) and 2,300 calories.

(*iv*) B. G., United Provinces.

> Rice home-pounded, boiled 11 oz.; dhal in curries 4 oz.; mustard oil 2 oz.; vegetables 4 oz.; sugar 2 oz.; tea, etc.
>
> This diet furnishes 1,200 μg. of vitamin B₁ (400 International Units) and 2,450 calories.

The essential differences in these diets consist of (*a*) the kind of cereal consumed, wheat atta being the cereal of the Punjabi diet and rice of the Bengalee and the U.P. diets; (*b*) the chief source of protein is meat in the Punjabi diet, fish and dhal in the Bengalee diet, in addition to milk in each case; (*c*) ghee is used for all cooking in the Punjabi diet, and mustard oil in the Bengalee diet. Subjects (*i*), (*ii*), and (*iii*) belonged to the same economic class, while subject (*iv*) was from a lower one both socially and economically.

Twenty-four-hour samples of urine were obtained and vitamin B₁ was recovered by adsorption at pH 3·0 on Lloyds' reagent according to the technique used by Harris and Leong (*loc. cit.*). The assay of vitamin B₁ was made by the rat-growth method. Graded doses of the dried samples of activated Lloyds reagent were fed to animals which had stopped growing and were just beginning to fall in weight on an otherwise complete but vitamin-B₁-free diet. The animals used were from a well-controlled breeding stock and controls were used with each test. The control animals used showed an average of 10 g. weekly growth response on feeding 10 mg. of the International Standard vitamin-B₁ preparation. At least two animals were used to test each dose. The results are expressed in μg. of vitamin B₁, the International Units obtained from biological tests being converted to μg. according to the relationship: 1 International Unit = 3 μg.

The average daily excretion of vitamin B₁ of the four subjects when living on their normal diets is shown in Table I. The output in all the four subjects is over 84 μg. (28 International Units) and in one case it is as high as 228 μg. (96 International Units) per day. The output is approximately 5·9 to 19·0 per cent of the intake. According to the standard proposed by Harris and Leong (*loc. cit.*) all the four subjects should be in excellent nutritional condition with respect of vitamin B₁.

TABLE I.

Subject.	Weight.	Age.	Approximate daily intake of the vitamin (μg.).	Total volume of urine excreted (ml.).	Total daily output of the vitamin (μg.).	Output as percentage of the intake.
	st. lb.					
B. A. ..	11–7	35	1,425	700	84·0	5·9
R. C. G. ..	8–4	28	1,350	1,100	132·0	9·9
G. R. ..	9–6	29	1,275	1,250	114·0	8·9
B. G. ..	8–0	58	1,200	2,000	228·0	19·0

The subjects then changed to a low vitamin-B₁ diet. Home-pounded and half-milled rice was replaced by polished rice and atta by white flour. Vegetables were somewhat reduced but the caloric value of the diet was maintained. The subjects remained on this low vitamin-B₁ diet for three days before the assays of the output in the urine were started. The results are summarized in Table II. There was a striking fall in the vitamin-B₁ excretion, the daily output being only 25·5 μg. to 36·0 μg. (8·5 to 12·0 International Units) which represents 3·1 to 5·7 per cent of the total intake.

TABLE II.

Subject.	Day of experiment.	Approximate daily intake of the vitamin (μg.).	Total volume of urine excreted (ml.).	Total daily output of the vitamin (μg.).	Output as percentage of the intake.
B. A.	1	600	..	..	..
	2	600	..	..	..
	3	600	..	..	..
	4	600	750	25·5	4·25
	5	600	748	27·0	4·50
R. C. G.	1	645	..	..	..
	2	645	..	..	..
	3	645	..	..	..
	4	645	1,000	33·6	5·20
	5	645	1,500	36·0	5·58
G. R.	1	840	..	..	..
	2	840	..	..	..
	3	840	..	..	..
	4	840	1,600	26·1	3·10
B. G.	1	588	..	..	..
	2	588	..	..	..
	3	588	..	..	..
	4	588	2,040	33·6	5·70

From the low vitamin-B₁ diet the subjects again changed to their normal diet and in addition took supplements of vitamin B₁ 600 μg. to 1,200 μg. (200 to 400

International Units) per day in the form of tablets of clay activated by adsorption from a concentrate of rice polish extract. The results of this experiment are shown in Table III :—

TABLE III.

Subject.	Day of experiment.	Total intake of the vitamin.		Total volume of urine excreted (ml.).	Total daily output of the vitamin (μg.).	Output as percentage of the intake.
		In food (μg.).	As supplement (μg.).			
B. A. ..	1	1,425	+ 600	..	..	..
	2	1,425	+ 600	760	54·0	2·66
	3	1,425	+ 600	765	68·4	3·37
	4	1,425	+ 1,200	1,200	78·0	2·97
	5	1,425	+ 0	1,250	29·7	2·08
R. C. G.	1	1,350	+ 600	..	..	..
	2	1,350	+ 600	1,500	45·0	2·30
	3	1,350	+ 600	1,500	126·0	6·30
G. R. ..	1	1,275	+ 600	..	..	..
	2	1,275	+ 600	1,700	68·4	3·64

It will be noticed that low intakes of vitamin B₁ for only 5 to 6 days had appreciably depleted the tissues and the output of the subjects could not reach the normal level until 3 to 4 days after maintaining them on a vitamin-B₁-rich diet. In one of the subjects (B. A.) the output rapidly declined when the vitamin supplement was stopped.

These results are in agreement with the observations of Harris and his colleagues. The amount of vitamin B₁ excreted in the urine depends upon the dietary intake and the previous dietary history of the subject. It represents however a small percentage of the total intake. When the intake is low, the percentage excretion is lower than that on a high vitamin intake. This might be a physiological adjustment on the part of the organism to conserve the stores in the face of a declining supply. The daily output of these subjects was higher than the output of normal persons at Cambridge reported by Harris. The ingestion of large amounts of vitamin B₁ had a marked diuretic effect in subject B. A. (Table III).

Since the diet of a large proportion of the population in India contains excessive amounts of carbohydrates, it was decided to investigate the effect on vitamin-B_1 output of a high carbohydrate and a high fat diet. A preliminary experiment by one of the subjects (R. C. G.) by inclusion in his diet of one pound of tapioca per day showed a marked decline in vitamin-B_1 excretion, while the ingestion of large amounts of butter somewhat tended to increase the output.

SUMMARY.

The urinary excretion of vitamin B_1 by four healthy subjects living on different Indian diets has been studied. Their normal daily output was found to be 84·0 μg. to 228 μg. (28 to 96 International Units) which represented 5·9 to 19·0 per cent of the intake.

A change in the diet for six days resulting in lower intakes of vitamin B_1, considerably reduced the daily output, and the normal level of excretion was not reached until 3 to 4 days after returning to a normal vitamin-B_1-rich diet including supplements of 600 μg. to 1,200 μg. (200 to 400 International Units) of vitamin B_1.

The ingestion of large doses of vitamin B_1 produced a marked diuretic effect in one of the subjects.

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STUDIES IN HUMAN METABOLISM.

Part II.

CALCIUM AND PHOSPHORUS METABOLISM IN INDIANS ON RICE AND ON WHEAT DIETS.

BY

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DIET surveys in a number of countries have revealed that average diets tend to show deficiency of calcium in terms of the standard advocated by Sherman (1920a). In general, where average or typical diets are concerned, a deficiency of calcium is more likely to occur than deficiency of any other mineral element. In India, cereals like rice and wheat usually constitute the bulk of the dietary and, except in certain areas, milk does not enter largely into the diet (Wright, 1937). In such circumstances the question of calcium intake and its adequacy assumes an even greater importance.

No metabolism studies designed to throw light on the calcium and phosphorus requirements of Indians have hitherto been carried out, and it remains uncertain whether the standards advocated by Sherman (1920a) and Leitch (1937), which are based on observations in Europe and America, are fully applicable in countries in which the diet is largely vegetarian and contains a preponderance of cereals. The problem of calcium and phosphorus metabolism in human beings consuming

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such diets requires separate investigation. In this connection reference must be made to Mellanby's (1921, 1925) observation that a high cereal diet had a deleterious effect upon the calcification of the bones and teeth in dogs and rats. The greater the proportion of cereal in the diet, the greater was the degree of rickets produced in Mellanby's experiments. Some cereals appeared to cause more severe rickets than others; these differences appeared to have no relation to the calcium or phosphorus content of the cereals. These observations of Mellanby were later confirmed by Holst (1927), Green and Mellanby (1928), and Thomas and Steenbock (1936) on rats and dogs. Although cereals form an important part of the diet of the human race, mineral metabolism experiments in human beings with cereals are surprisingly limited in number. Burton (1929) studied the influence of two cereals, wheat and oat, upon the retention of calcium and phosphorus in children and adults.

Deficiency of phosphorus appears less likely to occur in India than deficiency of calcium. Cereals and pulses contain fair amounts of phosphorus and the total phosphorus content of a largely cereal diet may satisfy the Sherman (1920b) maintenance requirement. Reference should be, however, made to the work of Bruce and Callow (1934) who suggested that the rachitogenic action of cereals might be due to the relatively high proportion of phytin phosphorus (40 to 64 per cent of the total phosphorus) which the cereals contain. They found that phytin phosphorus was not well absorbed by the rat and McCance and Widdowson (1935) observed that phytin was not well absorbed from human intestine, 36 to 63 per cent being excreted unchanged in the fæces. According to Bruce and Callow the so-called anticalcifying action of cereals may be due to the fact that the phytin present lowers the availability of calcium by binding it as a calcium salt which is at least as insoluble as calcium phosphate. Pulses also contain as high a percentage of phytin as cereals.

Poor vegetarians in India derive their calcium and phosphorus mostly from cereals and vegetables and not relatively inconsiderable amounts from the drinking water. There is no general agreement on the extent to which vegetable calcium is assimilated by living beings. Blatherwick and Long (1922) feeding cabbage, celery, spinach, and similar vegetables, and Rose (1920) experimenting with carrots found that calcium from vegetables was quite equal to milk calcium in the degree to which it was utilized in human adult maintenance. On the other hand Sherman and Hawley (1922) found that in children the calcium of milk was more efficiently utilized than vegetable calcium; Boas (1926) and Fincke and Sherman (1935) reported that spinach fed to rats was not favourable to calcium retention. McClugage and Mendel (1918) found that calcium of spinach and carrots was poorly assimilated by dogs.

The object of the present investigation was to determine the calcium and phosphorus requirements of Indians as suggested by balance studies on Indian subjects. Calcium and phosphorus retention when the amounts of rice or wheat in the diet were gradually increased and the difference in the retention of these minerals when either cereal formed the main item of the diet were also studied. The experimental diets were vegetarian and contained varying amounts of parboiled milled rice or whole wheat (atta). In addition they included vegetables (200 g.), pulse (25 g.), fat (25 g. butter-fat and mustard oil), and 100 g. sugar;

these supplements provided small and constant amounts of calcium and phosphorus. The energy value of all diets was kept constant by incorporating nitrogen-free sago which contains very little calcium and phosphorus. Milk was omitted from all the diets except one.

EXPERIMENTAL.

The experiments were conducted on three healthy young men, G. C. N. (19 years) and S. N. D. (24 years) each weighing 49 kilo, and B. C. R. S. (22 years)—one of the authors—weighing 54 kilo. S. N. D. and G. C. N. came from very poor village families and had lived on diets typical of those consumed by poor villagers. They lived with, and were kept under the strict supervision of one of the authors (M. N. B.). They realized the importance of the investigations and co-operated whole-heartedly in the work.

The composition and the calcium and phosphorus contents of the diets (as determined by us) are indicated in the tables. One type of cereal only was used during one series of experiments. The experimental subject was first given the diet containing the smallest amount of cereal; the quantity of cereal was then gradually increased keeping the total amount of food and its calorific value constant. Thus, diet I containing the lowest amount of cereal, was taken for six days, then without break diet II, and then diet III, both for six days. The experimental subject then took diet III for a further period of six days and then returned to diet II and then to diet I. One series of experiments with rice or wheat on one experimental subject thus lasted 36 days. In each series of experiments the cereal and the pulse were taken from the same stock every day, and aliquots of vegetables taken daily were pooled for analysis. The experimental subjects were given carefully weighed diets which they consumed *in toto.* The calcium content of drinking water was determined and taken into consideration.

For each diet, which was taken for six days, urine and fæces excreted on the last three days only were quantitatively collected, the first three days being considered as a preliminary period to avoid any effect produced by the previous diet. Urine was collected quantitatively for 24-hour periods and preserved with toluene to which some chloroform solution of thymol was added. The total volume of urine excreted each day was noted and then made up to a suitable volume for estimation. In order to be sure that the collection of urine was complete, the amount of creatinine eliminated daily was estimated and found to be uniformly constant. The fæces for the three days were collected together since the daily dry weight of the fæces was found to vary considerably. Fæces were marked by carmine. These were preserved with a small quantity of glacial acetic acid, dried over a water-bath with frequent addition of alcohol, thoroughly powdered, weighed, and preserved in the refrigerator in stoppered bottles.

The calcium in fæces and foodstuffs was determined by McCrudden's method (1911-12) and in urine by the method of Shohl and Pedley (1922). The phosphorus in urine and fæces was estimated by titration with uranium acetate according to Pincus (1859) and Malot (1887).

The experimental results are set out in Tables I to IX.

DISCUSSION.

Maintenance requirement of calcium and phosphorus.—Maintenance requirements of these elements can be deduced from the total amount excreted when the subject is just in a state of ' balance '. Sherman assumed that when output of calcium and phosphorus in any given experiment equals or nearly equals intake the output is a measure of requirements. If, with a given intake, there is a net loss of these elements from the body, intake is inadequate and requirements correspond to the sum of intake and loss. If, on the other hand, retention of the elements occurs, intake is considered to be in excess, and requirements correspond to intake minus the amounts retained. These assumptions are open to the objection that the results arrived at will to a considerable extent depend on the range of intake and by extending the range in one or other direction can be raised or lowered at will. If, however, we consider only those experiments as denoting requirements in which intake and output are nearly the same, we can make a reasonable assessment of requirements. Figures relating to the subjects at the points at which calcium and phosphorus balance was nearly attained are summarized in Table VII.

Mean calcium requirements, based on the above assumptions, re-calculated in terms of an adult of 70 kilo body-weight, are as follows: 0·226 g. (G. C. N.), 0·571 g. (S. N. D.) and 0·301 g. (B. C. R. S.) on rice diets and 0·226 g. (G. C. N.) and 0·661 g. (S. N. D.) on atta diets. The mean of all these results is 0·383 g. In all 28 experiments were performed on three individuals and the maintenance requirement has been calculated from 24 experiments in which calcium balance was nearly obtained. In 97 experiments Sherman (1920b) found daily calcium outputs ranging from 0·27 g. to 0·82 g. per 70 kilo body-weight with an average value of 0·45 g. Leitch (*loc. cit.*) has calculated calcium requirements from 400 metabolism records of non-pregnant non-lactating women by a graphical method. She finds a calcium requirement of 0·55 g. daily but the application of Sherman's method of calculation to the data utilized by her gives the value 0·415 g.

Of our three experimental subjects, G. C. N. comes from a poor family and in his case retention of calcium at low intakes may indicate that the skeleton was partly depleted of calcium before the investigation began ; Rottensten (1938) has shown that retention may be more efficient in such circumstances. Kelly and Henderson (1929-30) also found retention at low intakes in the case of negro prisoners in Africa. On the other hand, B. C. R. S., who also shows at a low calcium requirement, is a University graduate, comes from an upper middle-class family, had no history of malnutrition and is very healthy.

As regards. phosphorus, the average maintenance requirements, calculated in the same way, were as follows per 70 kilo : 1·063 g. (G. C. N.) and 1·091 g. (S. N. D.) on rice diets and 0·810 g. (G. C. N.) and 1·041 (S. N. D.) on wheat diets. In the case of B. C. R. S., the intake of phosphorus on the rice diets was rather high so that a considerable retention took place, and the output was higher than one would expect near balance point. If the phosphorus requirement is calculated from output with these high intakes, B. C. R. S. shows a phosphorus requirement of 1·417 g. per 70 kilo. The mean phosphorus requirement based on the average values obtained in 13 experiments in which balance was nearly obtained in two individuals works out at 1·001 g. for an adult weighing 70 kilo. If the requirements

of B. C. R. S. calculated from positive balance data be taken into consideration, the average requirement becomes 1·084 g. Sherman (1920*b*) has presented data relating to 95 phosphorus metabolism experiments performed on a number of individuals; he found that phosphorus outputs ranged from a minimum of 0·52 to a maximum of 1·20 g. with an average of 0·88 g. of phosphorus per 70 kilo of body-weight per day. Experimenting with 10 male subjects aged 32 to 70 years, Owen (1939) found a phosphorus requirement of 1·20 g. per day, a value also reported by Orr and Clark (1930).

The availability of calcium and phosphorus in rice and wheat.—Wheat contains about twice as much calcium and phosphorus as rice. Sago which replaced wheat and rice weight for weight in the diets containing lower amounts of cereal contains about as much calcium as rice and very little phosphorus. Thus, as intake of rice increased the total intake of calcium remained practically constant, while the percentage of calcium derived from rice increased. Intake of phosphorus, on the other hand, increased as more rice was consumed. With the wheat diets intake of both calcium and phosphorus, as well as the percentage of either derived from wheat, rose as the amount of wheat in the diets increased. On the rice diets G. C. N. showed a lower retention of calcium as the amount of rice in the diets was increased and a positive calcium balance at low intakes became a negative balance when 600 g. of rice was consumed; this roughly corresponds to the amount present in a typical poor rice-eaters' diet. S. N. D. was in negative calcium balance at all levels of rice intake but the net loss gradually increased with increase in the level of rice intake. It is thus quite obvious that rice is not only poor in calcium but that its calcium is also poorly utilized. On a typical middle-class Bengalee rice diet containing fish, B. C. R. S. was in positive calcium balance; the large intake of calcium in drinking water, which was derived from a tube-well, may have influenced the result. Increase in the intake of wheat increased retention of calcium, and on a wheat diet roughly corresponding to that consumed by wheat-eaters of the poorer classes, G. C. N. was in positive and S. N. D. in slightly negative balance. The fact that for the same intake of calcium G. C. N. was in positive calcium balance at lower levels of rice intake and in negative balance at higher levels is probably to be explained by the change in Ca/P ratio from 1/3·9 to 1/6·8. At the same unfavourable ratio of Ca/P (1/6·2) the experimental subject was in positive calcium balance on wheat diets because at the same ratio the levels of calcium and phosphorus intake were higher. Shohl (1936) observed that with rats as the levels of calcium and phosphorus in the diet increased at a given Ca/P ratio, both serum calcium and phosphorus and the percentage of bone ash increased and rickets and tetany diminished. The toxamin of Mellanby (1921, 1925) is probably not responsible for the negative calcium balance on rice diet for, according to the experiments of Mellanby (1937) himself, while maize and oat cause a very great deformity in the rib bones of rats and, therefore, contain appreciable amounts of the anticalcifying toxamin, rats fed on wheat or rice showed no bone deformity.

Rice and atta are rich in phosphorus and typical rice or atta diets (600 g. rice or atta), even without the addition of any milk, maintain the experimental subjects in phosphorus balance.

The Ca/P ratio in both the rice and atta vegetarian diets containing 600 g. of cereal, which correspond roughly to those consumed by poor wheat and rice-eaters,

is about $\frac{1}{6}$—an unsatisfactory ratio. The level of calcium intake is also low ; in this respect the wheat diet has the advantage and is to be preferred. Basu and Basak (1939) have shown that an average wheat diet contains about 74 g. protein, while an average rice diet contains only 54 g. The replacement of at least a part of the rice in the rice-eaters' diet by wheat is greatly to be desired. It is a welcome sign that middle-class people of Bengal and South India, two of the most important rice-eating parts of India, are gradually tending to consume wheat at one of their daily meals. So far as the requirement for calcium in itself is concerned, calcium salts like lactate might with advantage be habitually added to human food, either separately or by admixture with table salt but, as Sherman (1920a) observes, ' It would probably be more difficult to persuade people generally to make such additions than to teach a more liberal use of foods naturally rich in calcium '. Some vegetables are good sources of calcium but the availability of vegetable calcium is still a disputed question. The most important and useful supplement in this respect is milk, which besides being rich in calcium and phosphorus is a source of proteins of high nutritive efficiency and of vitamin A. Reference may be made to the results obtained with milk supplement in the case of B. C. R. S. and recorded in Table III. On a typical Bengalee diet containing rice, fish, pulse, and vegetables without milk the intake of calcium was 0·28 g. (this intake was influenced by the high calcium content of the drinking water from a tube-well which supplied 0·16 g.) and the experimental subject was just in calcium balance. On the addition of 466 g. milk, the intake of calcium rose to 0·86 g. the Ca/P ratio changed to 1/2·2 and a considerable retention of calcium took place. It may be pointed out that of the 584 mg. calcium and 498 mg. phosphorus supplied daily in the form of milk to the subject already in positive balance, 126 mg. calcium and 85 mg. phosphorus, calculated on an average of three days, were retained daily, the retention gradually falling off.

Channels of excretion.—It is well known that after absorption both calcium and phosphorus, especially the former, are excreted not only in the urine but also in the fæces. The percentages excreted in the urine and fæces are noted in Table VIII. Of the total phosphorus elimination 35 to 60 per cent occurs through the urine, while of the total calcium elimination 9 to 40 per cent takes place through the same channel.

Effect of excess of calories on mineral metabolism.—Addition of excess of cane-sugar to a diet already adequate in energy value (*vide* Table IX) increased the elimination of calcium in urine, while the fæcal calcium, as also urinary and fæcal phosphorus, remained practically constant. Reference may in this connection be made to the observation of Speirs and Sherman (1936) that within the limits of experimental error calcium utilization was similar on diets containing equivalent amounts of different forms of carbohydrate.

Protein intake and calcium metabolism.—Conner and Sherman (1936) observed that increasing the protein content of a diet containing a nearly minimal adequate level of calcium from 14 per cent to 18·8 per cent resulted in more rapid growth and also in an increased rate of calcification in young rats. In our experiments on adults, the level of protein in the diet appears to have no effect on calcium metabolism. Thus, in Table I (G. C. N.) as the amount of rice in the diets was increased from 250 g. to 600 g. the dietary nitrogen increased from 4·45 g. to 8·42 g. at a nearly

constant calcium intake of 0·147 g. and this had no favourable effect on calcium retention. Also with S. N. D. (Table II) an increase in rice intake increased the dietary nitrogen from 4·17 g. to 6·99 g., average intake of calcium being 0·213 g. ; no simultaneous improvement in calcium retention, however, occurred, the Ca/P ratio becoming worse.

SUMMARY.

1. Twenty-eight calcium and phosphorus metabolism experiments have been carried out on three individuals consuming vegetarian diets based on rice and whole wheat. The maintenance calcium requirement, deduced from the average of twenty-four experiments in which balance was nearly obtained, was found to be 0·388 g. per 70 kilo body-weight per day. The maintenance phosphorus requirement, deduced from the mean of 13 experiments in which equilibrium was nearly attained was calculated to be 1·001 g. per day per adult weighing 70 kilo.

2. Typical vegetarian rice diets (containing 600 g. rice and 0·15 g. to 0·20 g. calcium and with Ca/P ratio = $\frac{1}{6}$) fail to maintain adults in calcium balance. Typical wheat diets (containing 600 g. wheat and 0·30 g. to 0·35 g. calcium and with Ca/P ratio = $\frac{1}{6}$) give better results probably because of a higher level of intake of calcium at the same unfavourable Ca/P ratio. Replacement of a portion of rice by an equal weight of sago which contains the same percentage of calcium as rice but much less phosphorus brings the experimental subject, in some cases, into positive calcium balance. Replacement of a part of rice in the rice-eaters' diet by wheat is desirable.

3. The addition of 466 g. of milk to a rice and fish diet has a favourable effect on calcium balance and brings the Ca/P ratio to 1/2·2 and the calcium content of the diet to 0·86 g.

4. Both the rice and wheat diets kept the subjects in positive phosphorus balance.

5. Addition of excess of sugar to a diet already supplying sufficient calories increased the elimination of calcium in the urine, the fæcal elimination remaining constant.

6. Of the total calcium excretion 9 to 40 per cent occurs through the kidney, while of the total phosphorus elimination 35 to 60 per cent takes place through the same channel.

7. Increase in protein intake appears to have no effect on calcium metabolism in adults.

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TABLE I.

Calcium and phosphorus metabolism on rice diet.

Experimental subject—G. C. N.

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
I.										
250 g. rice ..	28	350	..	..	..	..	..	..	..	..
350 g. sago ..	34	21	..	..	..	..	..	..	..	..
200 g. vegetables ..	39	89	..	..	..	..	..	..	..	..
100 g. sugar ..	..	..	..	..	..	..	..	..	..	..
25 g. dhal (pulse) ..	10	107	9	..	..	..	352	..	..	..
25 g. fat ..	..	..	10	..	..	..	369	..	..	..
water ..	34	..	11	..	..	..	375	..	..	..
TOTAL ..	145	567	Mean 12	128	140	+5	365	271	636	−69

Ca/P = 1/3·9

TABLE I—*contd.*

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
II.										
400 g. rice ..	45	560	..	..	..	..	..	..	..	..
200 g. sago ..	19	12	..	..	..	..	..	..	..	..
200 g. vegetables ..	39	89	..	..	..	..	..	..	..	..
100 g. sugar ..	..	..	..	..	..	..	..	..	..	..
25 g. dhal ..	10	107	21	..	..	..	339	..	..	..
25 g. fat ..	..	..	25	..	..	..	449	..	..	..
water ..	34	..	14	..	..	..	354	..	..	..
TOTAL ..	147	768	Mean 20	126	146	+1	381	383	764	+4
Ca/P = 1/5·2										
III.										
600 g. rice ..	68	840	..	..	..	..	..	..	..	..
Nil sago	..	..	..	..	..	..	..	..	..	..

200 g. vegetables	39	89	:	:	:	:	:	:	:	:
100 g. sugar	:	:	:	:	:	:	:	:	:	:
25 g. dhal	10	107	25	:	:	:	423	:	:	:
25 g. fat	:	:	26	:	:	:	449	:	:	:
water	35	:	24	:	:	:	417	:	:	:
TOTAL ..	152	1,036	Mean 25	162	187	−35	430	563	993	+43
Ca/P = 1/6·8										

III.

600 g. rice	68	840	:	:	:	:	:	:	:	:
Nil sago	:	:	:	:	:	:	:	:	:	:
200 g. vegetables	39	89	:	:	:	:	:	:	:	:
100 g. sugar	:	:	:	:	:	:	:	:	:	:
25 g. dhal	10	107	26	:	:	:	383	:	:	:
25 g. fat	:	:	25	:	:	:	409	:	:	:
water	36	:	24	:	:	:	342	:	:	:
TOTAL ..	153	1,036	Mean 25	158	183	−30	378	455	883	+203
Ca/P = 1/6·8										

TABLE I—*concld.*

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily fæcal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily fæcal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
II.										
400 g. rice ..	45	560	..	..	..	..	..	..	..	..
200 g. sago ..	19	12	..	..	..	..	..	..	..	..
200 g. vegetables ..	39	89	..	..	..	..	..	..	..	..
100 g. sugar ..	..	..	..	..	..	..	..	..	..	..
25 g. dhal ..	10	107	20	..	..	..	406	..	..	..
25 g. fat ..	..	..	16	..	..	..	203	..	..	..
water ..	36	..	16	..	..	..	292	..	..	..
TOTAL ..	149	768	Mean 17	138	155	—6	300	484	784	—16

Ca/P = 1/5·2

I.

250 g. rice	28	:	:	:	:	:	:	:	:	:
350 g. sago	34	350	:	:	:	:	:	:	:	:
200 g. vegetables	39	21	:	:	:	:	:	:	:	:
100 g. sugar	:	89	:	:	:	:	:	:	:	:
25 g. dhal	10	107	16	:	:	:	293	:	:	:
25 g. fat	:	:	14	:	:	:	151	:	:	:
water	37	:	13	:	:	:	275	:	:	:
TOTAL ..	148	567	Mean 14	125	139	+9	240	304	544	+23

$Ca/P = 1/3\cdot8$

TABLE II.

Calcium and phosphorus metabolism on rice diet.

Experimental subject—S. N. D.

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
I.										
250 g. rice	61	391	..	..	..	..	..	..	..	..
250 g. sago	24	15	..	..	..	..	..	..	..	..
200 g. vegetables	59	70	..	..	..	..	..	..	..	..
100 g. sugar	..	..	..	..	..	..	..	..	..	..
25 g. dhal	25	105	190	..	..	..	267	..	..	..
25 g. fat	..	..	186	..	..	..	365	..	..	..
water	22	..	202	..	..	..	366	..	..	..
TOTAL ..	191	581	Mean 192	176	368	—177	332	315	647	—66
Ca/P = 1/3·0										
II.										
400 g. rice	97	626	..	..	..	..	..	..	..	..
100 g. sago	10	6	..	..	..	..	..	..	..	..
200 g. vegetables	59	70	..	..	..	..	..	..	..	..

100 g. sugar	:	:	:	:	:	:	:	:	:	:
25 g. dhal	25	105	120	:	:	:	369	:	:	:
25 g. fat	:	:	113	:	:	:	299	:	:	:
water	22	:	101	:	:	:	361	:	:	:
TOTAL	213	807	Mean 111	204	315	−102	343	394	737	+70
Ca/P = 1/3·8										
III.										
500 g. rice	121	782	:	:	:	:	:	:	:	:
Nil sago	:	70	:	:	:	:	:	:	:	:
200 g. vegetables	59	:	:	:	:	:	:	:	:	:
100 g. sugar	:	105	:	:	:	:	:	:	:	:
25 g. dhal	25	:	140	:	:	:	315	:	:	:
25 g. fat	:	:	145	:	:	:	304	:	:	:
water	22	:	153	:	:	:	319	:	:	:
TOTAL	227	957	Mean 146	287	433	−206	312	584	896	+61
Ca/P = 1/4·2										
II. Same as diet II above.			162	:	:	:	230	:	:	:
			164	:	:	:	316	:	:	:
			145	:	:	:	355	:	:	:
			157	256	413	−200	300	475	775	+32

TABLE III.

Calcium and phosphorus metabolism on rice, fish, pulse, and vegetables diet and the effect of supplementing the diet with milk.

Experimental subject—B. C. R. S.

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
I.										
400 g. rice	32	846	..	..	..	..	..	..	..	..
60 g. dhal	16	181	..	..	..	..	..	..	..	..
180 g. vegetables	33	100	70	159	229	+55	680	429	1,109	+288
120 g. fish	34	270	64	169	233	+46	722	366	1,088	+309
25 g. fat (butter-fat 5 g., mustard oil 20 g.).	..	..	51	183	234	+44	599	464	1,063	+334

water	165	..	95	135	230	+48	683	430	1,133	+284
TOTAL	280	1,307	Mean 70	162	232	+48	671	422	1,093	+304
Ca/P = 1/5·0										
I + 466 g. milk	584	498	121	396	517	+334	864	558	1,422	+473
			134	597	731	+121	836	622	1,458	+437
			93	695	768	+67	880	756	1,636	+259
TOTAL	864	1,895	Mean 116	566	679	+174	860	645	1,505	+389
Intake of Ca through water varied from 152 to 156 mg.										
Ca/P = 1/2·2										

Diet I was taken for seven days and urine and fæces collected on the last four days. Then diet I supplemented by 466 g. of cow's milk daily was taken for three days.

TABLE IV.

Calcium and phosphorus metabolism on whole wheat (atta) diet.

Experimental subject—G. C. N.

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
I.										
150 g. wheat　..	59	438	..	..	..	..	..	..	..	..
450 g. sago　..	44	27	..	..	..	..	..	..	..	..
200 g. vegetables　..	39	89	..	..	..	..	..	..	..	..
166 g. sugar　..	..	..	..	..	..	..	..	..	..	..
25 g. dhal　..	10	107	13	..	..	..	464	..	..	..
25 g. fat　..	..	..	19	..	..	..	249	..	..	..
water　..	19	..	20	..	..	..	273	..	..	..
TOTAL ..	171	661	Mean 17	159	176	−5	329	227	556	+105

Ca/P = 1/3·9

II.

400 g. wheat	157	1,168								
200 g. sago	19	12								
200 g. vegetables	39	89								
100 g. sugar	..	..								
25 g. dhal	10	107	27				517			
25 g. fat	..	..	18				559			
water	19	..	24				594			
TOTAL ..	244	1,376	Mean 23	160	183	+61	557	389	946	+430
Ca/P = 1/5·6										

III.

600 g. wheat	236	1,753								
Nil sago	..	..								
200 g. vegetables	39	89								
100 g. sugar	..	..								
25 g. dhal	10	107	15				520			
25 g. fat	..	..	22				586			
water	22	..	19				807			
TOTAL ..	307	1,949	Mean 19	154	173	+134	638	503	1,141	+808
Ca/P = 1/6·4										

TABLE IV—*concld.*

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
III.										
600 g. wheat	236	1,753	:	:	:	:	:	:	:	:
Nil sago	:	:	:	:	:	:	:	:	:	:
200 g. vegetables	39	89	:	:	:	:	:	:	:	:
100 g. sugar	:	:	:	:	:	:	:	:	:	:
25 g. dhal	10	107	17	:	:	:	557	:	:	:
25 g. fat	:	:	20	:	:	:	968	:	:	:
water	29	:	16	:	:	:	629	:	:	:
TOTAL ..	314	1,949	Mean 18	180	198	+116	718	633	1,351	+598
Ca/P = 1/6·2										
II.										
400 g. wheat	157	1,168	:	:	:	:	:	:	:	:
200 g. sago	19	12	:	:	:	:	:	:	:	:

200 g. vegetables	39	80								
100 g. sugar										
25 g. dhal	10	107	22				531			
25 g. fat			20				577			
water	26		23				485			
TOTAL	251	1,376	Mean 22	138	160	+91	531	377	908	+468

Ca/P = 1/5·5

I.										
150 g. wheat	59	438								
450 g. sago	44	27								
200 g. vegetables	39	89								
100 g. sugar										
25 g. dhal	10	107	11				263			
25 g. fat			14				319			
water	30		17				465			
TOTAL	182	661	Mean 14	98	112	+70	349	228	577	+84

Ca/P = 1/3·6

TABLE V.

Metabolism of calcium and phosphorus on atta diet (2nd series).

Experimental subject—G. C. N.

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
I.										
150 g. wheat ..	67·9	531·6	...	...	...	...	...	...	...	...
450 g. sago ..	50·8	22·5	...	...	...	...	...	...	...	...
200 g. vegetables ..	43·7	59·0	...	...	...	...	...	...	...	...
100 g. sugar ..	...	...	...	...	...	...	...	...	...	...
25 g. dhal ..	10·8	102	19·2	...	...	...	224	...	...	...
25 g. fat ..	...	...	13·4	...	...	...	437	...	...	...
water ..	33·3	20·7	25·5	...	...	...	476	...	...	...
TOTAL ..	206·5	735·8	Mean 19·3	118·5	137·8	+68·7	379	209	588	+147·8
Ca/P = 1/3·6										
II.										
200 g. wheat ..	90·6	708·8	...	...	...	...	...	...	...	...
400 g. sago ..	45·2	20·0	...	...	...	...	...	...	...	...

200 g. vegetables	43·7	59·0	:	:	:	:	:	:	:	:
100 g. sugar	:	:	:	:	:	:	:	:	:	:
25 g. dhal	10·8	102	17·8	:	:	:	402	:	:	:
25 g. fat	:	:	20·3	:	:	:	436	:	:	:
water	33·3	20·7	13·8	:	:	:	523	:	:	:
TOTAL ..	223·6	910·5	Mean 17·3	130	147·3	+76·3	453	212	665	+245·5
Ca/P = 1/4·1										

III.

400 g. wheat	181·2	1,417·6	:	:	:	:	:	:	:	:
200 g. sago	22·6	10·0	:	:	:	:	:	:	:	:
200 g. vegetables	43·7	59·0	:	:	:	:	:	:	:	:
100 g. sugar	:	:	:	:	:	:	:	:	:	:
25 g. dhal	10·8	102·0	22·7	:	:	:	764	:	:	:
25 g. fat	:	:	16·2	:	:	:	799	:	:	:
water	33·3	20·7	21·3	:	:	:	1,206	:	:	:
TOTAL ..	291·6	1,609·3	Mean 20·0	173·5	193·5	+98·1	923	368·8	1,291·8	+317·5
Ca/P = 1/5·5										

TABLE VI.

Metabolism of calcium and phosphorus on whole wheat (atta) diet.

Experimental subject—S. N. D.

Diet (daily intake).	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
I.										
300 g. wheat	150	777	..	..	..	..	..	..	..	..
200 g. sago	19	12	..	..	..	..	..	..	..	..
200 g. vegetables ..	59	89	..	..	..	..	..	..	..	..
100 g. sugar	..	..	..	..	..	..	..	..	..	..
25 g. dhal	20	112	73	..	..	..	270	..	..	..
25 g. fat	..	..	58	..	..	..	400	..	..	..
water	22	..	59	..	..	..	363	..	..	..
TOTAL ..	270	990	Mean 63	348	411	−141	344	614	958	+32
Ca/P = 1/3·4										
II.										
400 g. wheat	200	1,036	..	..	..	..	..	..	..	..
100 g. sago	10	6	..	..	..	..	..	..	..	..

200 g. vegetables	59	89								
100 g. sugar										
25 g. dhal	20	112	69				435			
25 g. fat			72				439			
water	22		73				382			
TOTAL ..	311	1,243	Mean 71	380	451	−140	418	843	1,261	−18

Ca/P = 1/4

III.

500 g. wheat	250	1,295								
Nil sago										
200 g. vegetables	59	89								
100 g. sugar										
25 g. dhal	20	112	69				352			
25 g. fat			79				316			
water	22		68				270			
TOTAL ..	351	1,496	Mean 72	401	473	−122	312	805	1,117	+379

Ca/P = 1/4·3

TABLE VII.

Maintenance requirement of calcium and phosphorus.

Showing the outputs of calcium and phosphorus at which balance was nearly attained in the experimental subjects.

Experimental subject.	RICE DIET.						ATTA DIET.					
	Ca-output (mg.).	Mean Ca-output or requirement (mg.).	Mean Ca-requirement per 70 kilo (mg.).	P-output (mg.).	Mean P-output or requirement (mg.).	Mean P-requirement per 70 kilo (mg.).	Ca-output (mg.).	Mean Ca-output or requirement (mg.).	Mean Ca-requirement per 70 kilo (mg.).	P-output (mg.).	Mean P-output or requirement (mg.).	Mean P-requirement per 70 kilo (mg.).
G. C. N. (49 kilo)	140 146 187 183 155 139	158	226	636 761 993 784 544	744	1,063	176 183 160 112 138 147 194	158	226	556 577	567	810
S. N. D. (49 kilo)	368 315 413 433 473	400	571	647 737 775 896	764	1,091	411 451	431	616	614 843	729	1,041
B. C. R. S. (54 kilo)	229 233 234 230	232	301	Too high phosphorus intake and balance.								

TABLE VIII.

Percentage elimination of calcium and phosphorus in urine and in faeces on rice and atta diets.

Experimental subject.	Diet number.	CALCIUM				PHOSPHORUS			
		RICE DIET.		WHEAT DIET.		RICE DIET.		WHEAT DIET.	
		Percentage elimination in urine.	Percentage elimination in faeces.	Percentage elimination in urine.	Percentage elimination in faeces.	Percentage elimination in urine.	Percentage elimination in faeces.	Percentage elimination in urine.	Percentage elimination in faeces.
G. C. N.	I.	9	91	10	90	57	43	59	41
	II.	13	87	13	87	50	50	59	41
	III.	13	87	11	89	43	57	56	44
	III.	13	87	9	91	45	55	53	47
	II.	11	89	14	86	38	62	59	41
	I.	8	92	12	88	44	56	60	40
S. N. D.	I.	52	48	15	85	51	49	35	65
	II.	35	65	15	85	47	53	33	67
	II.	38	62	:	:	38	62	:	:
	III.	34	66	15	85	53	47	28	72
B. C. R. S.	Rice and fish	30	70	:	:	:	:	:	:
	Rice, fish, and milk.	17	83	:	:	:	:	:	:

TABLE IX.

Effect of increase in intake of sugar and hence of calories on calcium and phosphorus metabolisms.

Experimental subject—G. C. N.

Diet (daily intake) and calories intake.	Ca (mg.).	P (mg.).	CALCIUM.				PHOSPHORUS.			
			Daily urinary Ca-output (mg.).	Average daily faecal Ca-output (mg.).	Total Ca-output daily (mg.).	Balance (mg.).	Daily urinary P-output (mg.).	Average daily faecal P-output (mg.).	Total P-output daily (mg.).	Balance (mg.).
I.										
3,000 calories	..	..	..	..	..	..	..	..	..	..
600 g. rice	83	1,197	..	..	..	..	..	..	..	..
Nil sago	..	..	..	..	..	..	..	..	..	..
200 g. vegetables	28	75	..	..	..	..	..	..	..	..
25 g. dhal	10	116	..	..	..	..	..	..	..	..
25 g. fat	..	..	16	..	..	..	347	..	..	..
100 g. sugar	..	..	21	..	..	..	344	..	..	..
water	36	..	17	..	..	..	351	..	..	..
TOTAL ..	157	1,388	Mean 18	115	133	+ 24	347	364	711	+ 677

Ca/P = 1/8·8

II. 3,500 calories; I + 125 g. sugar; Ca/P = 1/8·8	37	:	:	:	377	:	:	:
	60	:	:	:	419	:	:	:
	62	:	:	:	406	:	:	:
	Mean 53	116	169	− 12	401	386	787	+ 601
III. 4,000 calories; I + 250 g. sugar; Ca/P = 1/8·8	64	:	:	:	309	:	:	:
	77	:	:	:	243	:	:	:
	67	:	:	:	369	:	:	:
	Mean 69	107	176	− 21	307	350	657	+ 731

BASAL METABOLISM OF INDIANS.

A STUDY BASED ON THE EXAMINATION OF SIXTY NORMAL INDIAN MEN.

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WITH the growing importance of the determination of basal metabolism in the diagnosis and subsequent treatment of certain endocrine disturbances, demand arose for facilities for this work in Bombay. Since most of the hospitals lacked the necessary facilities, this Institute took up the work in 1927 and a considerable volume of clinical work was referred to it. It was soon realized that there was available no normal standard for Indians, except the few data reported by Mukherjee which averaged, according to Du Bois (1927), ' 9 per cent lower than generally accepted standards ', against which we could compare our findings in pathological conditions. It was the practice then, to use the standards for normals established by American workers on American subjects. The practice seemed to be founded on good experimental basis. At the time the American standards were worked out there was no indication but that they were applicable to other races and other climates. Some of the subsequent studies, however, on subjects of different races and on European residents in tropical and sub-tropical countries indicated significant differences from American standards. Yet the position was so confused that taking the same data two eminent authorities, Du Bois and Lusk, drew different conclusions. Du Bois (1927) concluded his discussion of the subject with the remark, ' There is evidence to indicate that there may be a rather large decrease in the inhabitants of some tropical countries '. Lusk (1928), on the other hand, summarized the evidence with the remark, ' On the basis of the whole of the evidence it does not appear wise to state that the influence of race or of a tropical climate may greatly reduce the basal metabolism. ' Data published

subsequently both in India (summarized in Table I) and abroad have not clarified the situation.

TABLE I.

Mean basal metabolic rates for normal Indian men in literature.

Name of observer.	Locality.	Number of subjects.	Average result expressed as percentage of an established standard used for comparison.	Type of apparatus used.
Mukherjee (1926) (quoted by Mukherjee and Gupta, 1931).	Calcutta	15	— 9 (Sanborn)	Douglas bag and Haldane gas-analysis apparatus.
Mukherjee and Gupta (*loc. cit.*).	,,	18	— 13·3 (A u b-D u Bois).	do.
Banerji (1931) ..	Lucknow	100	— 6·9 (European ?)	Benedict portable apparatus.
Krishnan and Vareed (1932).	Madras	54	— 12 (A u b-D u Bois).	Benedict-Roth apparatus with the Collin's Kymograph attachment.
Bose and De (1934) ..	Calcutta	30	— 0·7 (European and American ?).	Sanborn graphic metabolism apparatus.
Rahman (1936) ..	Hyderabad	32	—8·7 (Aub-Du Bois).	Sanborn Motor-Grafic Metabolism Tester.
Ahmad *et al.* (1938) ..	Calcutta	9	— 8·99 (A u b-D u Bois).	Douglas bag and Haldane gas-analysis apparatus.
Rajagopal (1938) ..	Coonoor	26	— 12·5 (A u b-D u Bois).	Benedict-Roth with Collin's Kymograph equipment.

The Indian data show a lowered heat production in normal Indian subjects. This observed lower metabolism is generally attributed to the influence of race and climate, but the study reported in this paper draws attention to another possible factor. We have noticed that in our subjects the daily (24-hour) excretion of nitrogen in urine seldom exceeded 7 g. and was very often as low as 4 g., while in the case of Europeans the usual figure given for 24-hour excretion of nitrogen is 12 g. to 18 g. This indicates that Europeans generally consume

much larger quantities of proteins than Indians do. The consideration emerges that the 'specific dynamic action' of proteins, particularly animal proteins, may be playing a decisive rôle in determining the level of heat production. It is true that in the determination of metabolism under basal conditions the specific dynamic action of proteins should play no part, yet it is possible that, in the standard conditions under which heat production is usually measured, the action of proteins may possibly form an important factor. In the case of Europeans consuming comparatively larger quantities of proteins, the specific dynamic action may produce a higher peak and the effect may well extend to the next morning ; while in the case of Indians habitually consuming comparatively small quantities of proteins, the rise in heat production due to protein action may be small, and the effect may come to an end in a much shorter period than 12 hours after the evening meal. In any case, the matter is of considerable importance and merits careful study. We, therefore, believe that before any final conclusion can be reached regarding the rôle played by climate and race on energy output, a considerable amount of controlled work will have to be carried out.

The first essential requirement of such studies would be the use by workers of a highly standardized accurate method of measuring the gas exchange. This requirement has not been given adequate attention by workers in India. Most of the workers have used a variety of apparatus employing closed-circuit methods measuring oxygen consumption alone. Two workers, who did use the open-circuit method, have employed the Douglas bag, with which it is apparently more difficult to obtain reliable results than with the other open-circuit methods (Carpenter, 1915). The multiplicity of methods employed for measuring the gas exchange and the different standards (sometimes unspecified) used for comparison have resulted in different workers arriving at basal metabolic rates for normal Indians varying from -0.7 per cent to -13.3 per cent. Further, measurements would have to be made on inhabitants of different sub-tropical and tropical regions using a standardized method for measuring the gas exchange. The question of the diets of subjects will require to be taken note of and determinations made while the subjects are subsisting on their usual diets and again after the same subjects have been put on diets rich in protein for suitable periods of time. In addition the basal metabolic rates of Europeans living in cold climates would have to be determined and then repeated on the same subjects after they move to sub-tropical or tropical regions immediately on arrival and then again at intervals after specified periods of stay in such areas. Level of protein consumption in their case also will have to be noted from time to time.

We could not conduct such extended investigations. We were interested in developing standards for clinical use. For our purpose it was enough to follow the suggestion on the matter made by Du Bois (1927), ' I should like to add that the man who studies the metabolism of patients should also study a fairly large number of normal controls, using exactly the same technic in order to ascertain the standards which show the best results under the given conditions of experimentation '. Though our work was undertaken for this limited objective, yet the greatest possible accuracy in the measurement of the heat production was attempted and full data are presented with a view to make this study serve a somewhat wider purpose.

GENERAL PLAN OF WORK.

Since in the case of patients neither previous training nor frequent repetition of determinations which are essential for determining strictly physiological standards is possible, we have sought to determine metabolism of normal Indian subjects under well-defined standard conditions.

As regards the choice of subjects, we preferred healthy men going about their daily work. So-called ' hospital normals ' were not used because such individuals, when they reach the hospital are in a low condition and tend to have a lower metabolism (Benedict, 1928). Further, as patients form a non-homogeneous group of individuals of all ages and from all classes of society, we have made our group of normal subjects representative by including individuals from different classes, and covering a wider range of age. Our normals were selected from college students and professors representing a highly educated class and a number of subjects from less educated classes. The ages varied from 20 to 49 years.

Regarding the measurement of heat production, we have adhered to the almost universal practice of making the determinations in the morning twelve to fourteen hours after the last meal with the subject lying motionless. Strictly speaking this is not the lowest or basal metabolism, and would be better designated as 'standard metabolism', as suggested by Krogh (1923, quoted by Du Bois, 1927).

Also from the very inception of our work we adopted the practice of utilizing the first determination made for the individual, unless at the time of the test it was noted that the respiration was unsatisfactory for reasons of restlessness and observable muscular tension. This is a matter of considerable importance and has recently been stressed by Boothby *et al.* (1936). They point out that the use of the term (basal metabolism) has led in recent years to the almost subconscious acceptance of the erroneous idea that, of any two or more determinations carried out on the same individual, the lowest or the lower group of such a series were the correct ones, and that the higher ones were vitiated by the presence of some unknown disturbing factor. Variability is a universal biologic phenomenon ; it must be taken account of by proper statistical methods and not excluded by a predetermined selection of results. Metabolism is a variable quantity that can be standardized, but not absolutely fixed. The idea that there is a single lowest metabolism for each individual is erroneous. They further point out that if many determinations are made on an individual and, of these, either the mean or lowest is used, the results will depend on the number of determinations which have been made for each case. Where a mean of several determinations is used, the value is slightly lower than the mean of first determination for many similar individuals, and of course this is true for the lowest determination. Therefore, if one departs from the practice of using a single determination made under standard conditions, the number of determinations should strictly be identical for each individual. That this condition would limit most investigations to few individuals is obvious, and therefore a standard should be built strictly on the single determination. We have used the first satisfactory determination throughout. Even in cases in which several observations were made either on the same or different days, the first satisfactory determination only was used for this study.

TECHNIQUE.

Our subjects came to the Institute at least 12 hours after the completion of their normal meal the previous evening. The actual tests for this study were made in all cases between 13 and 14 hours after the completion of the meal. Those who stayed in the Medical College Hostel, i.e., within a furlong of the Institute, came on foot but those who lived further away arrived in some conveyance at about 8 a.m. On arrival, the procedure was explained to the subjects to dispel their nervousness and gain their confidence, and they rested for at least half an hour horizontally on a couch before the test was started.

Because of the superiority of the gasometer open-circuit method over other types of open-circuit methods and its unquestioned accuracy, a chain-compensated spirometer (Tissot bell type) was used. The subject inspired the atmospheric air, the composition of which was checked from time to time, and expired into the spirometer through a mouth-piece, a series of tubes, and rubber flutter valves. The mouth-piece was found to be better than the face mask as the use of the latter was found to make subjects uncomfortable in the hot climate of Bombay. Besides, our experience showed that when a mouth-piece was used, leaks about the mouth could be more easily examined and excluded.

There is no objection to the use of normal standards determined under such conditions for evaluating metabolism in pathological conditions since all the patients for whom energy output determinations are desired can usually be sent to this laboratory, and determinations are made on patients under exactly the same conditions as those made in the case of our normal subjects. In any case the use of a non-portable apparatus is no impediment, because the patient who is too ill to be removed to a laboratory is not the type of case in which basal metabolic estimations are of value (Bailey, 1921).

After the period of rest it was ascertained that the body temperature was normal and the basal pulse and respiration rates were noted. Then the rubber mouth-piece was adjusted to the comfort of the subject and when he became accustomed to the mouth-piece, the nose-clip was applied and the subject was given some practice in breathing through the mouth. This enabled the subject to appreciate that there was no interference with respiration and helped in removing abnormalities of respiration. All leaks about the mouth and nose were examined and excluded.

When the subject had completely adjusted himself to the conditions and was breathing normally, he was connected with the spirometer during the inspiratory phase. After about five minutes, he was disconnected from the spirometer and the collected air was expelled. This procedure was repeated twice. It served a double purpose. It accustomed the subject to breathing into the spirometer and it washed the atmospheric air out of the instrumental dead space with expiratory air.

After a few minutes' rest in which the mouth-piece and nose-clip were removed, the subject was again connected with the spirometer for the actual test. The first few litres collected were expelled and the zero reading of the spirometer was taken. The subject was then connected with the spirometer by a quick turn of the

valve-cock during the inspiratory phase, the stop-watch was started at the same time and the collection begun. While the air was being collected, the respiration and pulse rates were recorded at frequent intervals. The amount of the gas collected in the spirometer per minute was recorded from the brass scale and this served as a good indicator of the regularity of respiration. The subject was requested to eschew all muscular activity during the test.

The duration of the test in our investigation varied from 10 to 15 minutes. At the selected time and during inspiration, the subject was quickly cut off from the spirometer by the turning of the cock, and the stop-watch was stopped at the same time. If the operator was satisfied that the breathing as regards depth and rate was normal throughout the entire period, the gas collected was used for analysis. The temperature of the spirometer gas, as recorded by a thermometer let through the top of the spirometer, was noted when it had practically (within 0·2°C.) come to the room temperature. No appreciable change in the volume of the gas, N. T. P. was noted whether it was read after 20 minutes or after one hour after the test nor was any significant difference in the composition of the gaseous mixture in the spirometer noted whether a sample was taken immediately or even as long as 30 minutes after the completion of the test. In all cases the volume of the expired gas was recorded and the sample taken for analysis after 20 to 25 minutes after the completion of the test. The spirometer was filled with water the previous evening so that it should have as nearly as possible the room temperature and thus help the air collected in the spirometer to acquire the room temperature quickly.

Next the standing height and the actual weight of the subject were recorded and the nude weight was calculated from the latter after deducting the approximate weight of clothes. This approximate weight was obtained by weighing a number of clothes similar to those used by the subjects. This method gave the nude weight correct to about half a pound, thus causing an error not more than about $\pm$ 0·2 per cent in the final result.

After the temperature and the volume of the gas collected in the spirometer and the barometric pressure had been recorded, some of the gas was let out to wash out the connecting tubes. A sample of the gas was then taken in a Bailey gas-sampling bottle. After the first sample was withdrawn and before the duplicate sample was taken, more gas was allowed to escape through the tubes to insure against errors from layering in the spirometer by the second sample being thus collected from a half-emptied gasometer. Carpenter and Fox (1927) have, however, shown that errors from layering are impossible when the subject has breathed normally and regularly throughout the test. It was our experience also, that there was no significant difference in the composition of the gaseous mixture in the two samples. The gas samples were analysed in a Haldane-Henderson gas-analysis apparatus, using a Henderson-Haldane-Bailey burette. From the values of CO_2 and O_2 obtained, the basal metabolism was calculated, using a five figure logarithmic table. The surface area was taken from the tables of Stoner (1926) drawn up on the height-weight formula of Du Bois and Du Bois (1916). The basal metabolism or basal metabolic rate was recorded as the percentile variation of the observed from the normal or predicted heat production.

As there is as yet no universally accepted normal standard, we have compared our findings with four different prediction standards. Three of these, namely, Aub-Du Bois (Sage) (1917), Harris-Benedict (1919), and Dreyer (1920) standards were selected because they are extensively used, while the fourth, namely Boothby-Berkson-Dunn (*loc. cit.*) standard, though also based on the surface-area formulation of Du Bois and Du Bois, was selected because the standard was worked out on a careful statistical study of a much larger number of subjects (639) than the original Aub-Du Bois standard. Of the three standards in wide use, the consensus of opinion seems to be in favour of the Aub-Du Bois standard which, though based on a small number of normal subjects, was found to indicate quite accurately the general changes with age that have been demonstrated during the last few years by the large series of Boothby (Du Bois, 1936). The standard is moreover less likely to fail when used on exceptional subjects of unusual build (Krogh, *loc. cit.*). Recently, Berkson and Boothby (1936) concluded their comparison of the estimation of basal metabolism from (1) a linear formula (Harris-Benedict) and (2) surface area (Du Bois and Du Bois) with the remarks : ' Our conclusions favoured the surface-area formulation, because of the consistency of its implications with observations and the relatively greater simplicity with which it can be used in the study of the change of metabolism with age or other variables '. Dreyer (*loc. cit.*), on the other hand, claimed preference for his method as in his opinion ' the formula $K = \dfrac{W^n}{C \times A^{0 \cdot 1333}}$ expresses the basal metabolism in an extremely satisfactory manner over a wide range of body, size, and age '. Better results were claimed by Dreyer when theoretical weight computed from the sitting height and chest circumference was used rather than actual weight, but the promised data on this point do not appear to have been published. Jenkins (1931), on the contrary, concluded that ' the Dreyer standards based upon observed weight seem on the whole to have the greatest advantages '. Stoner (1923) also found no advantage in theoretical over actual weight. Because of this possible lack of advantage and because of the greater possibility of error in the measurement of sitting height and chest circumference we have, in our calculations, used the actual weight.

Results.

Table II shows the results of our findings in 60 normal Indian men.

The basal pulse rate in our subjects average 67 beats per minute, the minimum being 51 and the maximum 85. The average given by Benedict (1928) for 24 out of 27 American subjects (the table of results does not give the pulse rate for 3 subjects) is 60. The minimum in this case was 43 and the maximum 75. This average according to Benedict was essentially the same as in the earlier series of Harris and Benedict of 136 men. Among the Indian investigators only three give basal pulse rates. Mukherjee and Gupta (*loc. cit.*) found the basal pulse rate to vary between 54 and 84, the average being 67. This average is the same as ours. Rahman (*loc. cit.*) found that the pulse rate during test averaged 64 beats, the range being 44 to 93. The average given by Rajagopal (*loc. cit.*) is 62, the range being 49 to 73. His figure for each subject is the average of nine counts in three tests.

Table II.

Basal metabolic rate determinations on sixty normal Indian men.

Serial number.	Age.	Standing height, cm.	Weight, kg.	Pulse rate, average.	Respiration rate, average.	Respiratory quotient.	Oxygen consumed per minute, c.c.	Heat produced per hour, cal.	Heat produced per sq. meter per hour, cal.	B.M.R. or percentile variation of the observed from the predicted heat production.			
										Aub-Du Bois (Sage) std.	Harris-Benedict std.	Dreyer std.	Boothby-Berkson-Dunn (Mayo Foundation) std.
1	20	170·2	49·8	68	14	0·808	211·7	61·1	39·0	− 2·6	0·0	− 0·7	− 6·3
2	20	162·6	44·9	56	23	0·891	180·0	53·1	36·6	− 8·6	− 6·5	− 9·2	− 12·1
3	20	170·2	49·6	61	17	0·917	197·4	58·6	37·3	− 6·7	− 4·3	− 4·9	− 10·2
4	21	168·9	49·6	62	19	0·814	203·2	58·7	37·8	− 4·4	− 3·0	− 3·7	− 8·3
5	21	170·2	50·8	63	21	0·804	191·2	55·2	34·9	− 11·7	− 10·3	− 10·7	− 15·3
6	21	171·5	79·0	60	24	0·880	278·2	81·8	42·7	+ 8·0	+ 5·0	+ 6·2	+ 3·5
7	21	173·3	47·1	56	14	0·813	204·3	59·5	38·4	− 6·9	− 8·0	+ 1·0	− 6·8
8	22	160·0	46·1	78	19	0·829	188·4	54·7	37·7	− 4·6	− 3·0	− 6·5	− 7·9
9	22	173·3	57·6	59	17	0·835	220·6	64·1	37·9	− 4·0	− 2·5	− 1·9	− 7·3

10	22	163·9	52·4	72	16	0·876	217·4	63·8	41·0	+ 3·7	+ 5·1	+ 2·5	+ 0·1
11	22	173·3	58·7	78	20	0·859	224·6	65·7	38·6	− 2·4	− 1·0	− 0·4	− 5·7
12	23	172·1	51·5	67	20	0·890	200·3	59·0	36·8	− 6·8	− 4·3	− 3·8	− 9·5
13	23	157·5	43·2	84	20	0·893	187·1	55·2	39·6	+ 0·2	+ 2·4	− 1·8	− 2·8
14	23	167·0	47·3	64	18	0·832	185·4	53·8	35·6	− 9·9	− 7·5	− 8·5	− 12·5
15	23	163·9	47·1	51	20	0·864	181·6	53·2	35·7	− 9·7	− 7·4	− 9·4	− 12·3
16	23	174·0	55·8	52	12	0·843	189·0	55·0	32·9	− 16·7	− 14·7	− 13·8	− 19·1
17	23	175·3	63·9	61	9	0·851	238·3	69·6	39·1	− 1·1	− 0·2	+ 1·8	− 4·0
18	23	168·9	72·5	73	13	0·828	226·7	65·8	36·0	− 8·9	− 9·9	− 9·6	− 11·6
19	23	173·3	56·6	61	15	0·913	195·5	58·0	34·6	− 12·5	− 10·6	− 9·9	− 15·0
20	23	165·1	56·6	73	13	0·790	208·6	59·9	37·0	− 6·3	− 5·1	− 6·8	− 9·1
21	23	174·0	53·4	72	18	0·876	218·6	64·2	39·2	+ 0·8	+ 1·6	+ 2·8	− 3·8
22	23	161·6	41·7	80	13	0·828	198·2	57·5	41·1	+ 3·9	+ 6·8	+ 4·2	+ 6·9
23	24	166·4	61·7	70	10	0·843	195·9	57·1	33·8	− 14·5	− 13·6	− 14·6	− 16·6

TABLE II—*contd.*

Serial number.	Age.	Standing height, cm.	Weight, kg.	Pulse rate, average.	Respiration rate, average.	Respiratory quotient.	Oxygen consumed per minute, c.c.	Heat produced per hour, cal.	Heat produced per sq. meter per hour, cal.	B.M.R. or percentile variation of the observed from the predicted heat production.			
										Aub-Du Bois (Sage) std.	Harris-Benedict std.	Dreyer std.	Boothby-Berkson-Dunn (Mayo Foundation) std.
24	24	172·4	50·3	60	14	0·833	165·9	48·2	30·3	− 23·2	− 20·7	− 20·1	− 25·1
25	24	178·1	58·6	63	15	0·809	219·3	63·3	36·5	− 7·7	− 5·1	− 2·7	− 10·0
26	24	180·3	54·6	63	19	0·857	200·4	58·6	34·5	− 12·8	− 9·7	− 6·8	− 14·9
27	24	169·5	54·9	60	13	0·800	192·1	55·3	34·0	− 14·0	− 11·9	− 12·2	− 16·2
28	25	177·8	73·9	82	20	0·733	220·8	62·5	32·7	− 17·3	− 16·8	− 14·0	− 18·9
29	25	159·0	37·8	72	14	0·872	153·0	44·9	33·8	− 14·4	− 11·2	− 13·6	− 16·0
30	25	152·4	45·6	62	20	0·838	169·1	49·2	35·3	− 10·6	− 8·3	− 13·8	− 12·4
31	25	157·5	47·3	70	16	0·847	187·8	54·8	37·8	− 4·3	− 1·7	− 5·8	− 6·2
32	25	169·5	50·5	66	16	0·813	215·7	62·3	39·7	+ 0·4	+ 3·9	+ 3·8	− 1·5
33	25	168·8	63·4	71	22	0·866	183·3	53·7	31·1	− 21·2	− 20·1	− 20·2	− 22·7

34	25	163·9	53·8	63	15	0·876	193·5	56·8	36·0	− 8·8	− 6·4	− 8·4	− 10·6
35	25	168·2	52·1	69	19	0·856	184·5	53·9	34·0	− 13·8	− 11·1	− 11·6	− 15·5
36	25	167·0	53·0	63	13	0·787	226·0	64·9	40·9	+ 3·5	+ 6·5	+ 5·4	+ 1·4
37	25	182·9	63·9	64	14	0·796	245·2	70·6	38·4	− 2·7	+ 0·1	+ 4·4	− 4·6
38	26	160·3	43·9	71	12	0·788	166·5	47·8	33·6	− 15·0	− 11·5	− 14·3	− 16·3
39	26	166·6	66·5	62	9	0·823	213·7	61·9	35·5	− 10·2	− 9·3	− 9·8	− 11·5
40	26	157·2	45·3	76	13	0·911	178·1	52·7	37·1	− 6·0	− 2·6	− 6·9	− 7·4
41	26	158·4	44·9	60	11	0·820	184·0	53·3	37·4	− 5·3	− 1·7	− 5·5	− 6·7
42	26	171·5	55·1	65	17	0·809	199·3	57·5	35·0	− 11·5	− 8·3	− 7·9	− 12·8
43	27	161·9	52·2	67	16	0·868	189·1	55·4	35·9	− 9·0	− 5·8	− 8·5	− 10·1
44	27	176·5	59·3	67	15	0·808	220·2	63·6	36·7	− 7·1	− 3·6	− 1·5	− 8·3
45	27	184·1	79·9	74	16	0·780	220·6	63·2	31·1	− 21·1	− 20·3	− 15·6	− 22·1
46	27	166·3	50·9	70	12	0·836	188·3	54·7	35·2	− 11·0	− 7·2	− 8·4	− 12·0
47	28	161·3	48·2	64	20	0·908	159·1	47·1	31·7	− 19·8	− 16·0	− 18·5	− 20·6

TABLE II—*concld.*

Serial number.	Age.	Standing height, cm.	Weight, kg.	Pulse rate, average.	Respiration rate, average.	Respiratory quotient.	Oxygen consumed per minute, c.c.	Heat produced per hour, cal.	Heat produced per sq. meter per hour, cal.	Aub-Du Bois (Sage) std.	Harris-Benedict std.	Dreyer std.	Boothby-Berkson-Dunn (Mayo Foundation) std.
										B. M. R. OR PERCENTILE VARIATION OF THE OBSERVED FROM THE PREDICTED HEAT PRODUCTION.			
48	28	161·9	53·0	62	18	0·820	208·8	60·5	39·0	− 1·4	+ 2·5	− 0·3	− 2·4
49	29	175·3	66·6	58	12	0·840	203·7	59·3	32·7	− 17·2	− 14·5	− 12·4	− 17·6
50	30	168·2	71·5	63	15	0·845	240·3	70·0	38·6	− 2·2	− 0·5	+ 0·3	− 2·4
51	30	179·1	85·9	66	14	0·831	295·0	85·7	41·7	+ 5·7	+ 5·9	+ 12·0	+ 5·4
52	30	165·1	50·0	83	15	0·754	227·9	64·9	42·3	+ 7·0	+ 13·1	+ 11·2	+ 6·7
53	30	165·1	42·5	85	14	0·713	188·6	53·1	37·0	− 6·2	0·0	− 1·2	− 6·5

54	— 7·2	— 5·6	— 3·0	— 8·2	36·3	58·5	202·0	0·822	12	66	58·4	161·3	34
55	— 13·4	— 9·7	— 7·5	— 14·4	33·8	51·0	174·6	0·853	13	66	48·4	163·9	34
56	— 15·7	— 13·6	— 12·0	— 17·2	32·7	54·8	184·9	0·910	12	60	61·8	163·9	36
57	— 6·1	— 0·8	— 0·7	— 8·8	36·0	60·9	208·5	0·857	13	82	59·3	170·5	39
58	— 8·8	— 10·3	— 8·4	— 11·6	34·9	61·9	212·1	0·851	17	76	75·3	158·1	40
59	— 10·6	— 4·1	— 4·6	— 11·8	34·0	58·0	202·1	0·786	16	60	58·6	174·0	42
60	— 10·5	— 10·6	— 4·0	— 13·8	33·2	51·5	182·1	0·732	15	74	55·5	157·4	49
Average	— 9·7	— 5·9	— 5·3	— 8·0	36·3	58·8	202·5	0·834	16	67	55·5	167·9	26·2

Three figures for average basal pulse rate for Western subjects quoted by Rahman (*loc. cit.*) are 62, 61·26 ± 6·73 and 61·4 ± 8·22. On this basis, the average pulse rate of Indians, except the value obtained by Rajagopal, is slightly higher than that of Western subjects.

The respiration rate varied from 9 to 24, the average being 16. Mukherjee and Gupta obtained an average of 15, the variation being from 10 to 20. Rahman's figures averaged 16·4, ranging between 9 and 31. Rajagopal's counts taken from the kymograph records average 16, the range being 9 to 25. These figures show that the average respiration rates obtained by different Indian workers are nearly the same.

The respiratory quotient varied from 0·713 to 0·917, the average being 0·834. The figures given by Mukherjee and Gupta range from 0·71 to 0·99, the average being 0·84, a figure very nearly the same as ours in spite of the wider range. Ahmad *et al.* found the range to be 0·72 to 0·87, the average being 0·81. All these figures are very close to the respiratory quotient, 0·82, generally accepted for the caloric value of oxygen to calculate the heat production from the measured oxygen consumption in a closed-circuit system.

The average oxygen consumption per minute was 202·5 c.c., the range being 153·0 c.c. to 295·0 c.c. The average of Mukherjee and Gupta is 186 c.c. (range 144 c.c. to 235 c.c.), that of Rahman 200·4 c.c. (range 164 c.c. to 245 c.c.), and that of Rajagopal 192 c.c. (range 170 c.c. to 214 c.c.).

The average heat production per hour per square meter was 36·3 cal., the range being 30·3 to 42·7 cal. The average of Mukherjee and Gupta is 34·26 cal. (range 27·19 to 39·40), that of Krishnan and Vareed is 34·8 cal. (range 29·4 to 42·0), that given by Rahman is 36·16 cal. (range 31·3 to 42·5), and that of Rajagopal is 34·4 cal. (range 31·5 to 37·0).

The comparison of our B. M. R. findings computed with four different standards, (*i*) Aub-Du Bois, (*ii*) Harris-Benedict, (*iii*) Dreyer, and (*iv*) Boothby-Berkson-Dunn (Mayo Foundation), are shown in a tabular form in Table III. The frequency distribution of the results by these standards is shown in Charts 1, 2, 3, and 4. It will be seen that the frequency distribution in the case of the Aub-Du Bois standard is the most symmetrical.

The mean, — 5·3 per cent by the Harris-Benedict standard, approaches more closely the zero of that standard, than is the case with other standards, but the total scatter about the average as well as the standard deviation are greater than those obtained with other standards. Thus, the Harris-Benedict standard gives the least relative prediction accuracy. Both the Aub-Du Bois and the Boothby-Berkson-Dunn standards are to be commended for their relatively better prediction accuracy; further, the total scatter about the average in the case of these standards is less than in the case of both the Dreyer and the Harris-Benedict standards. Though the average, — 8 per cent by Du Bois standard, is nearer the zero of the standard than the average, — 9·7 per cent, in case of Boothby-Berkson-Dunn standard, the latter standard is to be preferred for its greater range of applicability.

TABLE III.

Comparison of values obtained with four different prediction standards.

Standard.	Range, per cent.	Average, per cent.	Mean, per cent.	Median, per cent.	Standard deviation.	Percentage of subjects within significant variation.	Percentage of subjects within ±15 per cent of the average.	Extreme deviation from the average, per cent.	Total deviation, per cent.
Aub-Du Bois (Sage)	− 23·2 to + 8·0	− 8·0	− 8·0 ± 0·6	− 8·5 ± 0·7	6·9 ± 0·4	70·0	96·7	− 15·2 to + 16·0	31·2 (−) 23·2 to + 8·0
Harris-Benedict	− 20·7 to + 13·1	− 5·3	− 5·3 ± 0·6	− 6·3 ± 0·8	7·4 ± 0·5	71·7	96·7	− 15·4 to + 18·4	33·8 (−) 20·7 to + 13·1
Dreyer ..	− 20·2 to + 12·0	− 5·9	− 5·9 ± 0·6	− 6·8 ± 0·8	7·2 ± 0·4	65·0	96·7	− 14·3 to + 17·9	32·2 (−) 20·2 to + 12·0
Boothby-Berkson-Dunn (Mayo Foundation).	− 25·1 to + 6·7	− 9·7	− 9·7 ± 0·6	− 10·3 ± 0·7	6·7 ± 0·5	70·0	95·0	− 15·4 to + 16·4	31·8 (−) 25·1 to + 6·7

CHART 1.

Basal metabolic rates by the Aub-Du Bois standard.

CHART 2.

Basal metabolic rates by the Harris-Benedict standard.

CHART 3.

Basal metabolic rates by the Dreyer standard.

CHART 4.

Basal metabolic rates by the Boothby-Berkson-Dunn standard.

VARIATION IN THE BASAL METABOLISM OF AN INDIVIDUAL.

Even though, for reasons discussed previously, we used the first satisfactory determinations for the calculation of the mean, we also investigated the individual variation in the successive basal metabolic determinations. The variations noted in different determinations made on the same or on different days on the same individual are the sum total of three factors : (1) the true variation of the basal metabolism of the individual, (2) the experimental error of the apparatus, and (3) factors such as physical exhaustion on the previous day or unsuspected illness or unconscious increase in the muscular tension of the subjects. The knowledge of these variations is of tremendous importance from a practical stand-point when we attempt to interpret the results of any one determination (Du Bois, 1936).

(a) *Two determinations on the same day.*—We investigated 17 of our subjects on whom a second test could be made the same day in order to find the individual variation in the basal metabolic rate. The second tests were satisfactory as regards regularity of breathing, etc., in all cases. Our findings are shown in Table IV.

Comparison of the average of the means of two satisfactory determinations with the average of the first satisfactory determinations shows that the averages in the two cases are practically the same, namely,—6·3 per cent and —6·4 per cent by the Aub-Du Bois standard, and —4·0 per cent and —4·1 per cent by the Harris-Benedict standard. The individual minimum basal metabolic rate variation in the two tests on the same day is 0·4 per cent and the maximum 6·3 per cent by the Aub-Du Bois standard, the corresponding values by the Harris-Benedict standard being 0·4 per cent and 6·4 per cent. The minimum individual variation from the mean of two determinations is 0·2 per cent and the maximum is 3·2 per cent when compared with both the Aub-Du Bois and Harris-Benedict standards.

The minimum deviation in the oxygen consumption in the two tests is 1·7 c.c. and the maximum is 13·1 c.c. or 0·4 per cent and 3·3 per cent from their respective means. Our observed daily variations in oxygen consumption agree closely with the two data available in literature on Indian subjects. Rahman's (*loc. cit.*) minimum daily variation is 0 c.c. and the maximum is 15 c.c. or 0 per cent and 3·7 per cent from their means. Rajagopal (*loc. cit.*) stated that ' in almost all cases this (the basal oxygen consumption) was found to agree within 5 per cent '. Griffith *et al.* (1929) stated that the extreme deviation of duplicate determinations on the same day ranged between 3·0 and 5·3 per cent from their means in their six subjects, while in our seventeen subjects the extreme deviation is 0·4 and 3·3 per cent from their means.

(b) *Determinations on different days.*—We also examined seven of our subjects on two or more days with a view to determine the individual variation in basal metabolic rate on different days. The first satisfactory determination on each day was taken for calculation. Our findings are given in Table V.

The total minimum basal metabolic rate deviation of an individual is 1·2 per cent (—10·6 per cent and — 9·4 per cent Aub-Du Bois, and — 8·3 per cent and — 7·1 per cent Harris-Benedict), the corresponding figures for maximum deviation being 9·6 per cent (— 16·7 per cent and — 7·1 per cent) and 10·0 per cent (— 13·6 per cent and — 3·6 per cent) by the Aub-Du Bois and the Harris-Benedict standards,

TABLE IV.

Basal metabolic rate findings in two tests on the same day in seventeen of our normal subjects.

Serial number.	Respiratory Quotient.		Oxygen Consumed per minute, c.c.		Heat Produced per hour, cal.		Basal Metabolic Rate.			
							Aub-Du Bois.		Harris-Benedict.	
	Observed.	Mean.	Observed.	Mean.	Observed.	Mean.	Observed.	Mean.	Observed.	Mean.
3	0·917 0·910	0·914	197·4 200·3	198·9	58·6 59·3	59·0	− 6·7 − 5·5	− 6·1	− 4·3 − 3·1	− 3·7
4	0·814 0·808	0·811	203·2 190·1	196·7	58·7 54·9	56·8	− 4·4 − 10·7	− 7·6	− 3·0 − 9·4	− 6·2
6	0·880 0·867	0·874	278·2 291·1	284·7	81·8 85·3	83·6	+ 8·0 + 12·7	+10·4	+ 5·0 + 9·7	+ 7·4
9	0·835 0·802	0·819	220·6 218·1	219·4	64·1 62·9	63·5	− 4·0 − 5·9	− 5·0	− 2·5 − 4·4	− 3·5

Table IV—*concld.*

Serial number.	Respiratory Quotient.		Oxygen consumed per minute, C.C.		Heat produced per hour, Cal.		Basal Metabolic Rate.			
							Aub-Du Bois.		Harris-Benedict.	
	Observed.	Mean.	Observed.	Mean.	Observed.	Mean.	Observed.	Mean.	Observed.	Mean.
11	0·859	0·849	224·6	223·7	65·7	65·3	− 2·4	− 3·1	− 1·0	− 1·7
	0·839		222·8		64·8		− 3·7		− 2·3	
19	0·913	0·890	195·5	199·3	58·0	58·8	− 12·5	− 11·4	− 10·6	− 9·5
	0·867		203·0		59·5		− 10·2		− 8·3	
21	0·876	0·873	218·6	213·9	64·2	62·8	− 0·8	− 3·1	+ 1·6	− 0·7
	0·870		209·1		61·3		− 5·3		− 2·9	
26	0·857	0·869	200·4	199·4	58·6	58·5	− 12·8	− 13·0	− 9·7	− 9·9
	0·880		198·3		58·3		− 13·2		− 10·1	
33	0·866	0·870	183·3	186·4	53·7	54·7	− 21·2	− 19·9	− 20·1	− 18·8
	0·873		189·4		55·6		− 18·5		− 17·4	
34	0·876	0·884	193·5	194·6	56·8	57·3	− 8·8	− 8·1	− 6·4	− 5·7
	0·891		195·6		57·7		− 7·4		− 5·0	

No.														
35	0·856	0·844	0·850	184·5	191·4	188·0	53·9	55·7	54·8	−13·8	−10·9	−12·4	−11·1	−8·1	−9·6
36	0·787	0·825	0·806	226·0	218·9	222·5	64·9	63·5	64·2	+3·5	+1·2	+2·4	+6·5	+4·1	+5·3
37	0·796	0·764	0·780	245·2	242·7	244·0	70·6	69·3	70·0	−2·7	−4·5	−3·6	+0·1	−1·7	−0·8
42	0·809	0·809	0·809	199·3	197·6	198·5	57·5	57·0	57·3	−11·5	−12·2	−11·9	−8·3	−9·1	−8·7
43	0·868	0·897	0·883	189·1	186·8	188·0	55·4	55·1	55·3	−9·0	−9·5	−9·3	−5·8	−6·2	−6·0
48	0·820	0·830	0·825	208·8	219·9	214·4	60·5	63·8	62·2	−1·4	+4·1	+1·4	+2·5	+8·2	+5·4
54	0·822	0·744	0·783	202·0	211·4	206·7	58·5	60·0	59·3	−8·2	−5·8	−7·0	−3·0	−0·5	−1·8
AVERAGE ..			0·846			210·5			61·4			−6·3			−4·0

TABLE V.

Basal metabolic rate findings on different days in seven of our subjects.

Serial number.	Date of examination.	Respiratory Quotient. Observed.	Mean.	Oxygen consumed per minute, c.c. Observed.	Mean.	Heat produced per hour, cal. Observed.	Mean.	Basal Metabolic Rate. Aub-Du Bois. Observed.	Mean.	Harris-Benedict. Observed.	Mean.
27	24-9-27	0·800	0·824	192·1	196·0	55·3	56·8	— 14·0	— 11·7	— 11·9	— 9·5
	14-11-27	0·845		197·4		57·5		— 10·6		— 8·4	
	26-3-28	0·827		198·6		57·6		— 10·4		— 8·2	
30	2-5-28	0·838	0·868	169·1	169·0	49·2	49·5	— 10·6	— 10·0	— 8·3	— 7·7
	16-5-28	0·897		168·8		49·8		— 9·4		— 7·1	
44	28-9-27	0·808	0·844	220·2	207·7	63·6	60·5	— 7·1	— 11·9	— 3·6	— 8·6
	20-3-28	0·879		195·2		57·4		— 16·7		— 13·6	
49	10-2-28	0·840	0·828	203·7	217·2	59·3	63·0	— 17·2	— 12·6	— 14·5	— 9·8
	24-2-28	0·849		219·5		64·0		— 10·6		— 7·6	
	8-3-28	0·854		220·6		64·4		— 9·5		— 6·4	
	13-3-28	0·872		215·8		63·3		— 11·1		— 8·0	
	20-8-28	0·793		209·8		60·3		— 15·4		— 12·2	
	21-9-28	0·798		212·7		61·2		— 14·1		— 10·9	
	31-3-30	0·809		230·3		66·5		— 10·7		— 8·3	
	9-4-30	0·812		225·5		65·2		— 12·5		— 10·1	
50	21-4-28	0·845	0·803	240·3	252·1	70·0	72·7	— 2·2	— 0·2	+ 0·5	+ 1·4
	10-4-30	0·793		252·0		72·5		— 1·3		+ 0·2	
	14-4-30	0·771		264·1		75·5		+ 2·8		+ 4·4	
56	25-10-27	0·910	0·824	184·9	194·5	54·8	56·4	— 17·2	— 15·4	— 12·0	— 9·5
	22-3-28	0·815		192·5		55·7		— 15·1		— 9·2	
	2-4-30	0·770		200·9		57·4		— 14·5		— 8·6	
	6-3-31	0·800		199·8		57·6		— 14·6		— 8·3	
60	7-7-30	0·732	0·722	182·1	177·4	51·5	50·1	— 13·8	— 16·2	— 4·0	— 6·7
	10-7-30	0·712		172·7		48·6		— 18·6		— 9·4	
AVERAGE			0·816		202·0		58·4		— 11·1		— 7·2

respectively. The maximum deviation of the individual basal metabolic rate determinations from their average is ± 4·8 per cent by the Aub-Du Bois standard, the corresponding figure by the Harris-Benedict standard being ± 5·0 per cent.

Much larger variations have been observed. In one of the two extreme cases of Benedict (Du Bois, 1936) the maximum variation from the average was − 8·4 per cent to + 9·6 per cent (total variation 18 per cent according to square meter of body surface) during a period of 12 years and in the other the maximum variation was − 3·8 per cent to + 2·4 per cent (total variation 6·2 per cent) over a period of 16 years. Du Bois himself during 22 years varied from − 11·6 per cent to − 1·8 per cent (the total variation being 9·8 per cent).

Of the Indian investigators, Rahman's (*loc. cit.*) total minimum variation in basal metabolic rate on different days is 0·3 per cent (− 4·3 per cent and − 4·0 per cent) the corresponding value for maximum variation being 15·7 per cent (0·0 per cent and − 15·7 per cent) by the Aub-Du Bois standard.

As regards the variation in oxygen consumption per minute for an individual on different days, the minimum and maximum variation in our subjects were 0·3 c.c. and 26·3 c.c. Rahman's figures show the corresponding figures to be 4·0 c.c. and 36·1 c.c. Rajagopal's figures for the lowest oxygen consumption on two days show that the minimum variation is 1 c.c. and the maximum is 15 c.c. It is not clear whether these lowest oxygen consumptions represent tests made on two days only or whether the tests were made on several different days and which of these two determinations were selected for comparison. Benedict's (1935) oxygen consumption varied between 221 c.c. and 237 c.c. during the period of 29 out of 33 days, the maximum variation being 16 c.c. If four days when he was upset by an automobile accident which involved a member· of his family are taken into account, the figures were 221 c.c. and 255 c.c., the difference being 34 c.c. It is to be noted here, that Bose and De (*loc. cit.*) found little variation in the results when tests were made on the same subject on different occasions.

The maximum individual variation in the respiratory quotient on different days was 0·91 to 0·77. The average of the mean respiratory quotients for each subject was 0·816, a value closely approximating the value, 0·82, used in closed-circuit methods. The variation in the case of Benedict (1935) was 0·78 to 0·88 in 33 consecutive days.

OUR FINDINGS COMPARED WITH THOSE OF OTHER WORKERS.

A comparison of our findings with those of other Indian workers presents some difficulties. These must be stated for a correct understanding of the differences in the results recorded. Firstly, there is the question of the methods employed for measuring the gas exchange by different workers. We have employed the open-circuit method using a Tissot gasometer. This method measures gas exchange with a high degree of accuracy and has the great merit of making evident at once that something is wrong with the results if the technician has not been accurate (Du Bois, 1936). Only two other groups of workers, Mukherjee and Gupta (*loc. cit.*), and Ahmad *et al.* (*loc. cit.*), used the open-circuit method but they employed a Douglas bag, with which it is more difficult to get reliable results. All other workers employed the closed-circuit method. employing a uniform respiratory quotient of 0·82. This practice introduces an error of 1 to 2

per cent (Du Bois, 1936). For this reason alone our results are not strictly comparable to those of other Indian workers.

Secondly, the basal metabolic rate varies according to whether the first satisfactory determinations are used for its calculation, or whether the lowest readings of two or more determinations carried out on the same or different days, or the average readings of such determinations, are employed. As stated above we have employed the first determinations for our calculations. Mukherjee and Gupta and Ahmad *et al.* do not specifically state whether they used the averages or the lowest readings for their calculations, but it appears that they employed the averages of the successive readings for the calculation of their rate. Rahman, employing a closed-circuit method, used the averages of several readings whenever possible. Krishnan and Vareed, and Rajagopal, using the same type of closed-circuit apparatus. used the lowest of the three oxygen consumption determinations made on the same or different days.

In addition to the difficulties stated above a comparison with the work of some workers is made impossible by their omission to state the normal standard or standards which they employed to calculate their percentile variations. Banerji (*loc. cit.*) gives his basal metabolic rate to be ' 6·9 per cent below the European standard ', without specifying to which standard he was referring, there being no universally accepted European standard. Bose and De (*loc. cit.*) came to the conclusion that in the case of Indian subjects ' the basal metabolism does not essentially differ from that of the European and American standards '. These authors also do not specify to which standards they were referring. Though in absence of the knowledge of the standard used it is not possible to assess the value of their findings, yet it may be noted that the findings of these workers differ from those of all other workers in (1) that in the case of their Indian subjects they did not find any difference from the 'American and European ' normal standards, and (2) that the tests made on the same individuals (normal) on different occasions under similar conditions gave uniformly identical results.

Subject to the remarks made above, it will be seen that in the case of the Du Bois standard our mean basal metabolic rate of — 8·0 per cent is close to the figures — 8·7 per cent and — 8·99 per cent, given by Rahman (*loc. cit.*) and Ahmad *et al.* (*loc. cit.*), respectively. It is to be noted that the subjects of these workers came from two totally different localities. Rahman worked in Hyderabad (Deccan), while Ahmad *et al.* carried out their determinations on Bengalees in Calcutta. The mean basal metabolic rates, — 13·3 per cent, — 12 per cent, and — 12·5 per cent, given by Mukherjee and Gupta, Krishnan and Vareed, and Rajagopal, respectively, are lower than our finding. In the case of the Harris-Benedict standard our mean rate, — 5·3 per cent, is close to that, — 5·79, given by Ahmad *et al.* (*loc. cit.*), while the other available figures, — 10·8 per cent, — 6·8 per cent, and — 8·9 per cent, given by Krishnan and Vareed, Rahman, and Rajagopal respectively are lower than our finding. In the case of the Dreyer standard our rate, — 5·9 per cent, is close to the rate, — 6·16 per cent, given by Ahmad *et al.* who appear to have calculated the heat production from the actual weight as we have done. The only other workers, Krishnan and Vareed who compared their findings with the Dreyer standard, calculated their heat production from the weight calculated from sitting height, and obtained a rate of

— 11·9 per cent. It is to be noted that this rate of theirs is practically the same as they obtained with the Du Bois standard, i.e., — 12 per cent.

DISCUSSION.

The mean basal metabolic rate for normal Indian male subjects given by different workers varies from — 0·7 per cent to — 13·3 per cent. If the figures of Banerji (*loc. cit.*) and Bose and De (*loc. cit.*), who do not state what normal standard they used for comparison, are not taken into consideration, the variation of the rate narrows down to — 8·0 per cent to — 13·3 per cent by the Aub-Du Bois standard. These different rates are reported on the basis of work carried out at Bombay, Calcutta, Madras, Hyderabad (Deccan), and Coonoor. The question, therefore, arises whether these different rates actually represent different levels of basal metabolism among normal subjects living in widely separated localities. There is little evidence, however, to make one believe that such is the case. Mukherjee and Gupta (*loc. cit.*) working at Calcutta found a basal metabolic rate of — 13·3 per cent for normal Bengalee males, while Ahmad *et al.* (*loc. cit.*) who also worked at Calcutta found a rate of — 8·99 per cent for Bengalees. Both used Aub-Du Bois standard for comparison. We must, therefore, look for some other factor to explain the observed differences.

We have stated above that different workers have used different methods of measuring the gas exchange. We have used the open-circuit method employing a Tissot gasometer. Other workers have used closed-circuit methods, except two who used the open-circuit method employing a Douglas bag. Though it is true that the Tissot apparatus is unquestionably superior on grounds of accuracy of measurement, the recorded differences are too large to be entirely attributed to the use of different methods of determination. In the case of closed-circuit determinations in which a uniform respiratory quotient of 0·82 is employed for calculations, the error due to this practice would only amount to 1 to 2 per cent.

To a great extent, as we shall show below, the observed differences are due to whether the readings of the first satisfactory determinations, or the lowest readings of two or more determinations carried out on the same individual on the same or different days, or the averages of the readings of such determinations, were used for the calculation of the basal metabolic rates.

Our findings in the case of 17 subjects on whom two determinations were made on the same day show that the mean basal metabolic rate is — 7·6 per cent (Aub-Du Bois standard) when the lower determinations are used, while it is — 6·4 per cent when the first determinations are used. The mean based on the first determinations is thus higher than the mean calculated on the lower determinations by 1·2 per cent. It is to be noted that the mean calculated on the averages of the two determinations (— 6·3 per cent) is practically the same as the mean based on the first determinations (— 6·4 per cent). Similarly, the mean basal metabolic rate (— 11·7 per cent Aub-Du Bois standard) calculated on the first determination in the case of seven subjects on whom determinations were made on two or more days, agrees with the mean rate based on the averages of determinations (11·1 per cent). But the mean basal metabolic rate works out as — 13·8 per cent when calculated on the lowest readings. Thus, the mean calculated on the first determinations is higher by 2·1 per cent than the mean based on the lowest determinations.

Perhaps our cases are too few to establish correctly the difference between the means based on the first determinations and those based on the lowest readings, but a difference of about the same magnitude has been noted by a worker who worked with a very large series of subjects. Marjorie Smith (quoted by Jenkins, 1932) working in Chicago and using a portable Benedict apparatus made determinations on 1,126 men and 2,994 women. This worker found that the mean, if calculated on the averages of readings, was — 7 per cent (Aub-Du Bois standard), while it was — 9 per cent if calculated on the lower readings. Jenkins, using the same apparatus in the same locality on a series of 34 normal University freshmen, found a mean rate of — 5 per cent (Aub-Du Bois standard) calculated on the averages of determinations made on the same day. He observes, ' The difference between — 5 per cent and — 9 per cent (the figure obtained by Marjorie Smith) is in part accounted for by the fact that in my data on normal persons, successive determinations on a given day were averaged, while in the data of the clinic (Marjorie Smith) the lower reading was selected.' The Indian workers Krishnan and Vareed (*loc. cit.*), who measured basal metabolism of 58 normal subjects taking three readings as a rule, found the rate to be — 7 per cent (Aub-Du Bois standard) if the averages of the readings were used and a rate of — 11·6 per cent if the lowest readings of the three readings were employed.

These data show that the difference between the rates calculated on the averages of successive determinations and the rates calculated on the lowest of two or three readings are 1·3 per cent, 2 per cent, 4 per cent, and 4·6 per cent, giving an average difference of 3 per cent, and it may safely be assumed that rates determined on the lowest readings of successive determinations would be about 3 per cent lower than the rates calculated on the averages of two or three readings or on the first readings.

Our basal metabolic rate of — 8·0 per cent (Aub-Du Bois standard) calculated on the first readings, agrees closely with that of Rahman (— 8·7 per cent) and of Ahmad *et al.* (— 8·99 per cent) who employed the averages of successive readings for their calculation. Krishnan and Vareed's rate of — 7·0 per cent calculated on the averages of three successive readings is also close to our rate. This leaves Rajagopal's rate of — 12·5 per cent. He used the lowest readings of his successive determinations. His paper does not give data to permit of a rate being calculated on the averages of readings taken on the same day. But if we correct his rate, based on the lowest reading, by the value (3 per cent) given above, we get a rate of — 9·5 per cent. This amount of correction is not excessive as the correction figure employed is based on the lowest of two or three readings, while Rajagopal took the lowest reading of six determinations. It will thus be seen that the basal metabolic rates for normal male Indian subjects reported by different workers lie within the range of — 7 per cent and — 9·5 per cent. They agree closely with the rate of — 8·0 per cent found by us. Our rate has been determined by one of the most accurate methods under strictly standardized conditions and its difference of about ± 1 per cent from the rates found by other workers may well be due to the use by other workers of the closed-circuit method and a uniform R.Q. of 0·82.

The available evidence shows conclusively that the basal metabolism of normal Indian subjects is lower than that of the European and American subjects; and that the average lowering of the rate is 8 per cent by Aub-Du Bois standard. This

lowering of the basal metabolism is usually attributed to racial and climatic factors, but no satisfactory evidence has yet been put forward in support of this assertion. It seems more likely that the observed low basal metabolic rate is due largely to dietetic factors, i.e., to the relatively small amounts of proteins habitually consumed by the Indian people. Though conclusive evidence on the point is not available, a number of observations point towards it. Krogh and Linhard (1920) show that metabolism is decreased if the previous diet is low in protein and that the lowest rates are obtained when the respiratory quotient is between 0·80 and 0·94. Bierring (1931, quoted by Du Bois, 1936) confirmed this observation using a trained subject. Deuel *et al.* (1928) in a well-controlled experiment have shown that with the withdrawal of proteins from the diet the basal metabolic rate fell from — 9 per cent to — 20 per cent by Aub-Du Bois standard. The nitrogen excretion during this period had fallen from 9·73 g. to 3·92 g. per 24 hours. It is also held (Du Bois, 1936) that a large protein meal can exert a specific dynamic action for more than fourteen hours. Krishnan and Vareed (*loc. cit.*) found that the average basal rate of seven subjects on a high protein diet was + 1 per cent by Aub-Du Bois standard and of seven other subjects on low protein diet the average rate was — 19 per cent. These observations obviously refer to large differences in the quantities of protein consumed, the fact still remaining that in the case of our subjects, even when they consumed meat and other foods containing animal proteins, the nitrogen excretion in urine in 24 hours' periods seldom exceeded 7 g., while the average excretion in the case of European and American subjects on a mixed diet varied from 12 g. to 18 g. per day. In view of the facts cited it is not unreasonable to conclude that the observed low basal metabolic rate in the case of Indian subjects is very likely due to their habitual low consumption of protein.

SUMMARY.

1. The mean basal metabolic rate for normal Indians has been worked out by the study of heat production, under precisely specified standard conditions, of

Averages and ranges of variations in normal findings of sixty men.

Prediction standard used for comparison.	Mean, per cent.	Minimum, per cent.	Maximum, per cent.	Standard deviation.	Percentage of subjects within significant variation.
Aub-Du Bois (Sage)	— 8·0	— 23·2	+ 8·0	6·9 ± 0·4	70
Harris-Benedict	— 5·3	— 20·7	+ 13·1	7·4 ± 0·5	72
Dreyer	— 5·9	— 20·2	+ 12·0	7·2 ± 0·4	65
Boothby-Berkson-Dunn (Mayo Foundation).	— 9·7	— 25·1	+ 6·7	6·7 ± 0·5	70

60 normal Indian men.   Their ages ranged from 20 to 49 years.   The open-circuit method employing Tissot type gasometer was used for measuring the gas exchange and the first satisfactory determinations were used to establish the rate.   The findings are summarized above.

2.   It is shown that the mean basal metabolic rates reported so far in the literature for normal Indian men, either originally calculated from the averages of successive determinations or corrected to correspond to that basis if originally calculated from the lowest of several readings, lie between — 7·0 per cent and — 9·5 per cent by the Aub-Du Bois standard.   Our finding of — 8·0 per cent based on the first determinations is very close to these figures.   The small difference is due to the fact that other workers whose rates differ from ours employed closed-circuit methods for their determinations and used a uniform respiratory quotient of 0·82 for their calculations.

3.   It is suggested that the low basal metabolic rate in the case of normal Indian subjects (— 8·0 per cent by the Aub-Du Bois standard) is due to the very low protein content of the usual Indian dietary and is not due to racial or climatic factors as such.

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STUDIES ON HÆMOLYSIN OF COBRA VENOM.

Part I.

INVESTIGATIONS ON THE ISOLATION OF HÆMOLYSIN FROM COBRA (*NAJA NAJA*) VENOM.

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AMONGST the earlier workers who claimed to have separated hæmolysin from neurotoxin the following names might be mentioned. Kyes (1903) claimed to have separated hæmolysin from neurotoxin by combining it with lecithin and termed the resulting compound a lecithide. Later workers, however, have shown that an active hæmolysing agent (lyso-lecithin) is formed by the interaction of venom with lecithin, which can hæmolyse in the absence of both of them. Flexner and Noguchi (1905) separated hæmolysin by adsorption with appropriate red blood cells. But this method is unsuitable, since it requires the addition of unknown quantities of protein substances. Dunn (1934) observed that *crotalus-adamanteus* venom acting on cephaline produces intensely hæmolytic substances. He separated an active ' cephalinase ' from *crota-adamanteus* venom with strong hæmolytic but no proteolytic activity. The hæmolytic activity in this fraction was of the same order as that of cephalinase. Ganguly and Malkana (1936) and Ghosh and De (1937*a*) have shown that by treating cobra-venom solution with sodium chloride the whole of the hæmolysin is precipitated and this precipitate is free from neurotoxin. Ghosh and De (*loc. cit.*) purified it further by heating at 86°C., a faintly alkaline solution of the precipitate and finally by cataphoresis of the solution obtained after heat coagulation. For the same protein content the hæmolysin fraction so obtained was four times more active than the crude venom. In this paper the results obtained by the author in an attempt to purify the hæmolysin still further are recorded.

MEASUREMENT OF HÆMOLYTIC ACTIVITY.

Slotta *et al.* (1937) measured the hæmolytic activity of venoms by adding the solution of venom to a mixture containing buffer, lecithin, and horse red blood cell suspension. The minimum amount in γ which hæmolysed the mixture in two hours was taken as the lecithinase unit. The method employed by the author was similar to that of Slotta *et al.* Four c.c. of blood from the heart of guinea-pigs were taken in 1 c.c. sterile 10 per cent sodium citrate solution to which 15 c.c. buffered saline solution (8·1 g. NaCl + 1·19 g. anhydrous CH_3COONa adjusted to desired pH with acetic acid) were added and the mixture centrifuged to separate the cells. The cells were then washed four times with the same saline (60 c.c.). The solution of venom and all dilutions were made in saline of definite hydrogen-ion concentration. The reaction of the medium was adjusted within the range pH 7·4 to pH 7·6 because it has been found to be the optimum condition for hæmolytic activity. To 0·3 c.c. of solution of cobra venom were added 50 γ of the best quality of egg lecithin freshly emulsified in 0·2 c.c. of buffered saline and 1 c.c. of 5 per cent suspension of washed red blood cells of guinea-pigs and incubated at 37°C. Ten γ of crude venom requires about 30 minutes for the complete hæmolysis of the cells, and for convenience it was taken as the unit of hæmolysin. For determining the hæmolytic activity of different fractions, graded amounts of them were set up against 10 γ of crude venom and the tube in which hæmolysis occurred as in the standard, was noted.

FIRST METHOD OF PURIFICATION.

When methyl alcohol was added to a solution of cobra venom the major fraction of the protein was precipitated. This precipitate contained a small amount of hæmolysin, the major amount being present in the supernatant liquid. The precipitate formed was colloidal in nature and it was difficult to centrifuge it out. If the precipitation was effected in presence of sodium chloride, the precipitate formed was coarse and easy to centrifuge. Moreover the addition of the salt caused the precipitation of a greater amount of protein without much loss of hæmolysin. The optimum concentration of the salt in the solution of venom required for the precipitation of inert proteins was found by trial to be 8·0 per cent. Precipitation at different hydrogen-ion concentrations was also carried out to find out the conditions under which the maximum recovery of the hæmolysin with the maximum elimination of inert proteins occurs. It was observed that solutions adjusted to pH 2·6 to pH 3·0 gave the desired results.

(a) *The first stage of removal of inert proteins by precipitation with methyl alcohol.*

A one per cent solution of venom was prepared in 8·0 per cent sodium-chloride solution. Aliquot portions of it were transferred to different flasks and adjusted to different hydrogen-ion concentrations by the addition of minimum quantity of hydrochloric acid. Two c.c. fractions from each of them were transferred to a series of stoppered measuring cylinders. They were kept in the frigidaire for one hour ; after this period 12 c.c. of cold methyl alcohol were added to each of them.

After one hour the supernatant liquid was separated by centrifuging. The soluble fraction was dried in a vacuum desiccator. The protein content and the hæmolytic activity were determined in each fraction.

TABLE I.

One mg. of the crude venom contains 100 *units of hæmolysin.*

pH of the venom solution.	Protein in mg.	Hæmolysin units.
2·2	7·08	1,860
2·6	5·62	1,730
3·0	5·33	1,650
3·6	4·95	1,400
4·6	4·12	1,230
5·8	3·24	1,060

(b) Second stage of purification with acetone.

For further purification, the precipitate obtained from 100 mg. of venom by treatment with alcohol was taken up with 10 c.c. of water, adjusted to pH 5·0, and kept in the frigidaire for 30 minutes. Fifteen c.c. cold acetone were then added to the solution and kept for another 30 minutes in the frigidaire; this precipitated further portion of the inactive proteins. The precipitate was separated by centrifuging. The supernatant solution was again treated in the cold with 50 c.c. of cold acetone; the precipitate formed was sticky and adhered to the sides of the flask. The solution was centrifuged and the precipitate from the centrifuge tube and the flask was dissolved in 5 c.c. water and evaporated to dryness in a vacuum desiccator.

This fraction contained 10·45 mg. protein and 7,550 units of hæmolysin.

(c) Third stage of purification by adsorption on various adsorbents.

It has been shown in a previous paper (De and Ghosh, 1937) that Fuller's earth and Kieselguhr adsorb less of hæmolysin and more of protein from a solution of venom than many other adsorbents. Attempts were, therefore, made to remove the inert proteins by adsorbing them on such adsorbents. Other adsorbents tried were (1) Merck's charcoal, (2) barium carbonate, and (3) ferric hydroxide (negatively charged).

Starting with 100 mg. venom the final product obtained after acetone precipitation was dissolved in 10 c.c. of 0·9 per cent sodium-chloride solution and to this were added weighed amounts of each adsorbent suspended in 10 c.c. water and the

mixture shaken for one hour in the shaker. The adsorbent was then removed by centrifuging. The protein content and the hæmolysin units determined in the supernatant liquid separated from each adsorbent.

TABLE II.

Experiment number.	Adsorbent.		Amount of adsorbent in mg.	CONTENTS OF SUPERNATANT LIQUID.	
				Protein in mg.	Hæmolysin units.
1	Fuller's earth	..	200	8·14	7,040
2	Kieselguhr	..	200	8·45	7,550
3	Merck's charcoal	..	200	6·40	5,480
4	Barium carbonate	..	200	7·82	5,820
5	Ferric hydroxide	..	48	5·88	6,500

From Table II it is observed that using ferric hydroxide as adsorbent the maximum purification is achieved. By this method a purified hæmolysin fraction was obtained, the activity of which was, weight for weight, 11·06 times greater than that of the crude cobra venom.

SECOND METHOD OF PURIFICATION.

It has been shown by Ghosh and De (1937a) that the major portion of the hæmolysin can be separated from the neurotoxin when solid sodium chloride is added to a venom solution, so as to bring the concentration of the salt in the solution to 20 per cent. Further purification was possible by heating the product in a faintly alkaline solution. Attempts were made to find out more efficient methods of purification. It was found that if the precipitate obtained from 22 per cent solution of sodium chloride is dissolved in water and treated with metaphosphoric acid, the major part of the inert proteins was thrown down, leaving the hæmolysin in the solution.

PROCEDURE WHICH WAS FINALLY ADOPTED FOR THE PURIFICATION OF HÆMOLYSIN.

To 200 c.c. of 0·5 per cent cobra-venom solution was gradually added 40 g. solid sodium chloride. The mixture then kept in the thermostat at 37°C. for one hour. The precipitate was separated from the solution by centrifuging and was washed in the centrifuge tube with 20 per cent sodium-chloride solution. The precipitate was then dissolved in 100 c.c. of water. This fraction contained 388·6 mg. protein and 92,500 units of hæmolysin.

To the above 100 c.c. solution, 5 g. of metaphosphoric acid, freshly dissolved in 20 c.c. of water, were added and the mixture allowed to stand for 30 minutes at 37°C. for complete precipitation. The precipitate was separated by centrifuging. This precipitate contained a very small quantity of hæmolysin. As the phosphoric acid interferes with the estimation of hæmolysin it was removed as follows :—

To the supernatant liquid was added 5 c.c. of 10 per cent sodium tungstate and 5 c.c. of $\frac{2}{3}$ normal sulphuric acid and it was kept at 37°C. for 15 minutes. The precipitate was separated by centrifuging and washed thrice with a solution containing per every 100 c.c. 5 c.c. of 10 per cent sodium tungstate and 5 c.c. of $\frac{2}{3}$ normal sulphuric acid. The precipitate was then dissolved in water by the addition of N/5 NaOH, the reaction of the solution being finally adjusted to pH 9·2. The tungstic acid was removed by the addition of slight excess of barium chloride, the pH being maintained in the same region by the addition of barium hydroxide. · The supernatant liquid was separated from the precipitate by centrifuging and the excess Ba-ions removed by adding the requisite amount of sodium-sulphate solution. The solution was then evaporated to dryness. This product contained 111·7 mg. protein and 75,200 units of hæmolysin.

EXTRACTION WITH COLD PYRIDINE.

The above precipitate was dissolved in 5 c.c. water and then precipitated in the cold with 20 c.c. of pyridine cooled to 4°C. The precipitate formed was separated from the supernatant liquid by centrifuging and it was found to be inactive. The supernatant liquid was dried in vacuum. This procedure purified the hæmolysin a little further, e.g., 65·2 mg. protein was associated with 65,850 units of hæmolysin.

As it has been shown in the first method that ferric hydroxide adsorbs more of the inert proteins than hæmolysin, so the pyridine extract was treated with ferric hydroxide. Adopting the same method as described before the whole amount of hæmolysin was concentrated in a protein fraction which constitutes only 8·32 per cent of the crude venom. Therefore a purification of 11·3 times of the hæmolysin fraction has been effected.

This purified hæmolysin was subjected to cataphoric experiments with the object of purifying it still further. The apparatus and arrangements used were the same as described in a previous paper by Ghosh and De (1937b). No further purification of the hæmolysin fraction was, however, possible over the whole range of pH 2·0 to pH 9·6. The hæmolysin migrated to the cathode even when the reaction of the solution was pH 9·6 showing it to be basic in nature.

SUMMARY.

Hæmolysin of cobra (*Naja Naja*) venom has been purified by two different methods and by both the methods a concentration of eleven times of the hæmolysin fraction has been effected.

The first method consisted of precipitation with methyl alcohol, then fractional precipitation with acetone followed by the removal of the inert proteins by adsorption on ferric hydroxide gel.

The second method consisted of precipitation with 20 per cent sodium chloride and then precipitation by metaphosphoric acid and lastly extraction with cold pyridine.

ACKNOWLEDGMENT.

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OBSERVATIONS ON *P. PAPATASII* IN THE PESHAWAR DISTRICT.

Part I.

BY

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*(Sandfly Fever Inquiry, 1938-39, Peshawar Section,
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THE high incidence of sandfly fever during the hot weather amongst troops in Peshawar and adjoining areas on the North-West Frontier Province presents a problem of considerable importance to the medical authorities, and in recent years the disease and its vector, the *P. papatasii*, have been the subject of several special inquiries. An investigation was carried out in Peshawar in 1925 by a Commission, the results of which have been reported by McCombie Young, Richmond and Brendish (1926), and further investigations, chiefly on the bionomics of sandflies and the methods of their control, were made by Puri (1934, 1935) at Landi Kotal in the Khyber Pass, 29 miles from Peshawar.

There still remain many points to be cleared up with regard to sandfly fever in the area and, in association with an investigation into the virus of the disease, observations were again commenced in 1938 on points in connection with the epidemiology of sandfly fever, bionomics of sandflies, and control methods, an account of which is given in this communication.

EPIDEMIOLOGICAL OBSERVATIONS.

Sandflies were already present when the Inquiry was commenced on the 1st April, 1938, and as the first case of sandfly fever occurred on the 8th April it appeared that infective flies must have been present at the end of March. Cases continued throughout the hot weather up to the end of October. The number of sandflies decreased considerably by the end of October and on the 7th November an experienced collector obtained only 11 flies in upwards of one hour's work. Three days later, on the 10th November, no adult flies could be found.

A spot map was prepared to show the distribution of cases in 1937, and was maintained throughout 1938, and a study of their distribution showed certain irregularities in incidence which were the subject of further investigation.

In Gough Lines, occupied by the 16th Light Cavalry, there were, in 1937, 16 cases of the fever among the Sowars of the Regiment, of which 13 occurred in one Squadron (B). The Lines were inspected and no obvious cause for this noted, but on inquiry it was learned that, while the remainder of this Regiment is recruited from around Meerut and from Jaipur and Alwar States, B Squadron obtains its personnel from Jodhpore, which is situated well away from the ' sandfly areas ', as defined by Rogers and Megaw (1930). This was borne out in 1938, when the number of cases in the Regiment was 8, of which 6 came from B Squadron. This Squadron's stables lie about twenty yards from, and on the windward side of the men's barrack rooms, so that the ' animal barrier ' appears to provide incomplete protection. This fact, which endorses the views of Chandler (1936), is not unexpected, as *P. papatasii* is believed to be essentially, if not entirely, a human blood feeder.

A rather similar observation was made in Edwardes Lines, occupied by the 2nd-19th Hyderabad Regiment, where the figures, by Companies, were as follows : In 1937, A—13, B—3, C—3, D (S)—0, H.Q.—13 ; total—32. In 1938, A—6, B—4, C—4, D (S)—1, H.Q.—2 ; total—17. Here again an inspection on the ground provided no clue to the cause of the unequal distribution, and inquiry was made into the ' geographical factor '. It was learned that A Company and one-third of H.Q., who jointly provided three-quarters of the cases, consist of hillmen (Kumaonis), while the remainder of the Battalion come from the plains of the western part of the United Provinces.

In Connaught Lines, occupied by Nos. 15 and 29 A. T. Companies, R. I. A. S. C., rather more detailed investigations were carried out. These two Companies, each of a strength of 230 I. O. Rs., live in adjacent barrack buildings of precisely similar type and under identical conditions. As neither Company was in Peshawar in 1937, comparative figures for that year were not obtainable, but at an early date it was evident that cases were occurring in much larger numbers in No. 15 Company, i.e., by 20th May six cases, as compared with one in No. 29 Company. A squad of sandfly collectors was organized from the Companies, and carried out a survey over four days. The average number of flies, per hour, caught by each man in the two areas was as follows : No. 15 Company—18·7 ; No. 29 Company—15·0. These figures are higher than in other Lines similarly surveyed, but this was not due to any slackness in carrying out routine precautions which were, in fact, particularly strictly enforced in both Companies. The Lines are situated adjacent to the perimeter wire, and just outside are a number of houses of ' katcha ' construction, whose mud walls contain many cracks and would appear to afford ideal breeding places for sandflies. The slightly higher figure for No. 15 Company was insufficient to account for the unequal incidence of the fever, and the area was kept under observation for the rest of the hot weather. During the month of May, No. 15 Company was engaged on military training and No. 29 on station duties, which are less strenuous and involve less hardship on the men. These duties are interchanged at the end of every month. A record was kept of the date of onset of each case of the fever, to determine if this change of duties coincided with a change in the

incidence rate, and so suggested the existence of a 'physical strain' factor. A record was also kept for each case of his place of birth and length of service, in order to investigate the 'geographical factor', and also to detect any special tendency to the disease among either recruits or old soldiers.

The final figures showed a total of 16 cases in No. 15 Company, and 6 in No. 29, equivalent to 69·6 and 26·1 per mille respectively. The ratio per thousand for Indian troops in the whole of Peshawar was 17·6. Of these 22 cases, 20 were hillmen, mostly from the Kangra valley and Poonch State, while the remaining two were semi-hillmen, from the Hazara district. Their lengths of service varied considerably as follows : Up to 3 years—10 ; 4 to 6 years—*nil* ; 7 to 10 years—4 ; 11 to 13 years—8. The incidence during the two different types of duties was as follows : Military training—10 ; station duties—12. It would appear that the 'geographical factor' is the only one that has a direct bearing on the incidence of the fever, and the higher incidence in No. 15 Company seems to be explained by the fact that its personnel contains a larger proportion of men drawn from hill races.

It has frequently been noted that there is a high incidence of the fever in Gurkha battalions, when stationed in sandfly areas, and these observations suggest that this is probably equally true of other hill races who seem to enjoy a much lower degree of immunity than the plainsmen of Northern India. The exact reason for this immunity is still a matter of conjecture, and there is no evidence to show whether it is of an inherited or an acquired nature.

Among British troops the incidence, as was expected, was considerably higher than among Indians. The only problem presented was in Maude Lines, and is dealt with at a later stage in these notes.

OBSERVATIONS ON THE BIONOMICS OF *P. papatasii.*

Breeding of sandflies in the laboratory.

At first, efforts at breeding the flies in the laboratory were unsatisfactory and it was not until the Inquiry's insect collector had been sent to the Pasteur Institute, Kasauli, for ten days' special training, that this was carried out successfully. Two methods were used. One was similar to that used at the Calcutta School of Tropical Medicine and described in the report of the McCombie Young Commission, in which a wide-mouthed vessel of unglazed clay is half-filled with a mixture of desiccated rabbit fæces and dried earth. Above this is placed a layer of wet mud, which is incised crucially, and the vessel is placed in the sun for several hours. This causes the mud to bake dry and to crack along the lines of incision. The mouth of the vessel is then closed with a piece of butter muslin, under which one or more fed females are introduced. After some days these females will deposit their eggs in the recesses of the cracks. In the second method, fed females are preserved in an inverted test-tube, fitted with a plug of well-moistened cotton-wool, for some days until ovulation has taken place. The plug is then removed and the eggs are washed off, with a stream of water from a wash bottle, on to a circle of filter-paper, three inches in diameter. This paper is placed in a small shallow dish of unglazed pottery, and on it are placed a small quantity of desiccated rabbit fæces and a pinch of powdered dried blood. In both methods the requisite moisture is provided by standing the vessel or dish in a metal tray, containing well-moistened sand or coarse earth.

The former method provides conditions more nearly akin to Nature, and is to be preferred when flies are required in large numbers. The latter method makes it possible to observe the various steps of metamorphosis and enables specimens to be removed for examination at any desired stage.

Female flies were, on several occasions, fed on volunteers, for breeding purposes. It was observed that an interval of more than five minutes elapses between the cessation of feeding and the feeling of irritation at the site of the bite.

Effect of temperature on breeding.

The relation between the length of time required for complete metamorphosis of *P. papatasii*, and the environmental atmospheric temperature, was illustrated by observations on some artificially-bred flies. Several fed females were introduced into a breeding vessel on 20th July, and hatched out adults made their appearance on 20th August, i.e., after 31 days. During this period the temperature in Peshawar varied very little and the daily average range was maximum 100°F. to 107°F., minimum 80°F. to 84°F. A second batch of fed females were similarly put down on 28th August and hatched out adults did not appear until 25th October, i.e., after 60 days. A third batch, put down on 7th September, showed hatched out adults on 26th October, after 49 days. The temperature in Peshawar on 27th August was maximum 100°F., minimum 82°F., following which it dropped fairly steadily, and during the seven days immediately previous to 25th October, it declined from maximum 93°F., minimum 79°F. to maximum 80°F., minimum 51°F.

These observations bear out the remarks of Chandler that the time required for metamorphosis varies directly as the environmental temperature. The temperatures recorded above were obtained from the Royal Air Force Meteorological Office, Peshawar. Humidity readings were not obtained as the breeding vessels were kept surrounded by water, and their environmental humidity was probably considerably higher than that of Peshawar generally.

Height to which sandflies can fly.

This work was prompted by certain remarks of Chandler, to the effect that ' . . . Boyd's report of Officers in North-West India, being bitten by sandflies on a roof 40 feet above ground, appears to require confirmation'. Phillips (1923) states that, in Egypt, persons sleeping on the upper stories of buildings enjoy immunity from the bites of sandflies. In a personal communication Lieut.-Colonel Shortt has informed me that he has caught sandflies at the tops of trees in Assam.

The site selected for the work was a two-story barrack building occupied by the 8th Anti-Aircraft Battery, Royal Artillery. This Unit's Lines consist of two double-story buildings, placed end to end, each about 120 yards long and lying 150 yards apart. Their lower stories are about 20 feet high and the upper 25 feet. Their long axis runs north-east to south-west. Both floors have an outer verandah on the south-east side and the upper floor has, in addition, on the north-west side, an inner verandah on which men were sleeping.

At the commencement of the investigation, the strength of the Unit was about 210 B. O. Rs., of whom 180 were sleeping on the upper story and 30 on the lower.

Work was carried out on three lines, viz., the use of a simple form of light trap, the actual catching of sandflies by hand, and the observation of the incidence of the fever among the personnel sleeping on each floor.

The light traps consisted of ordinary electric bulbs and their shades, well greased with vaseline, and left burning all night on each of four successive nights (May 3rd and 4th to 6th and 7th, inclusive). The total catch on the upper floor was 2,360 insects, and on the lower 1,840, of which the numbers of *P. papatasii* were 129 and 111 respectively.

In September, as a final measure, another simple type of light trap was placed on an exposed platform at a height of 75 feet, on each of three nights. This trap consisted of a hurricane lamp, placed in the centre of a square board, on which four fly papers were fixed with drawing pins. On the three nights, out of a total of 780 insects caught, six were *P. papatasii* (two males and four females). The weather on each of these three nights was fine, dry, and warm.

The catching of flies by hand was carried out, morning and evening, over four days in the middle of May. The average number caught each day, expressed in 'man-hours', was: Upstairs 16, downstairs 10·6. The upper floor yielded a larger 'bag' on all days except the last, when there was a very considerable wind, and the majority of the flies appeared to be driven down to the comparative shelter of the lower floor.

At the conclusion of the season, figures for the incidence of the fever on both floors were obtained, and are set out below in tabular form. Nearly three-quarters of the personnel of the Battery left for the hills at the end of May, and its strength was reduced from 210 to 60. This hill party did not return until the 10th October, nearly four weeks after the last case of sandfly fever from this Unit was admitted to hospital. Table I shows the average number of men sleeping on each floor during each month, and the number of cases of the fever which occurred :—

TABLE I.

Month.	PERSONNEL SLEEPING.			
	UPSTAIRS.		DOWNSTAIRS.	
	Number of men.	Number of cases.	Number of men.	Number of cases.
April ..	180	1	30	1
May ..	180	9	30	0
June ..	60	4	..	..
July ..	60	2	..	..
August ..	60	2	..	..
September ..	60	2	..	..

The incidence rate among men sleeping on the upper floor did not differ markedly from that of other British Units in Peshawar. The figures are small, and in some degree inconclusive, on account of the absence of controls (in the form of men sleeping on the ground floor) during the four later months. They serve however to show that sandfly fever does occur among persons sleeping at a height of 20 feet above ground. This fact together with the knowledge that the flies themselves exist in large numbers at this height, and can be found at a height of 70 feet, indicates that there should be no relaxation of precautions on the part of individuals whose sleeping places are on roofs or upper stories.

Effect of wind on the flight of sandflies.

With the co-operation of Flight-Lieutenant L. H. Starr, Meteorological Officer, Royal Air Force, some work was carried out early in October, to ascertain what was the minimum wind speed against which sandflies could not fly. The apparatus used consisted of a horizontally placed glass cylinder, five inches long and four inches in diameter, one end of which was closed with mosquito netting. A stream of wind was blown through the cylinder and its velocity measured at either end by means of an anemometer. The mean of these two velocities was taken as the velocity within the cylinder. Single specimens of *P. papatasii* were released inside the cylinder, and their behaviour in relation to various wind velocities noted. It was found that the maximum speed against which a fly could just make some headway was in the region of 1·6 m.p.h., while the minimum speed against which it could not fly was in the region of 1·8 m.p.h. In practical language this last speed is approximately equivalent to the current of air produced by an ordinary table fan, revolving at its slowest speed, at a distance of six to seven feet. The mosquito netting at the end of the cylinder did not, of course, prevent the escape of the flies, but served to delay them sufficiently for it to be gauged whether they were making any headway against the wind. It is to be emphasized that this work was carried out under conditions rather different from those in Nature, i.e., the tests were performed in the day-time, when sandflies are possibly more sluggish than at night, and secondly no bait was provided to stimulate the flies to extra efforts. These facts should be borne in mind in the practical application of the results, and a margin of safety allowed.

CONTROL MEASURES.

It is becoming increasingly evident that of the various preventive measures in force in Peshawar and Landi Kotal, few are of greater importance than the proper supervision of unoccupied buildings, whether ruinous or otherwise. Ruined buildings, especially those built of mud, invariably contain large numbers of cracks and fissures which form potential breeding places for sandflies, and such buildings, if in the neighbourhood of occupied premises, should be either demolished, or very thoroughly renovated. Unoccupied buildings, although otherwise in a fair state of repair, if not overhauled periodically, are liable to develop cracks in their walls in which sandflies may shelter and breed. An example of this occurred in Maude Lines, occupied by the 27th-28th Medium Battery, R. A. It was noticed, in May,

that the incidence of the fever from the whole Battery was above the average of the station, and that the majority of the cases were occurring in one particular barrack room. Investigation showed that the most probable cause of this was the presence of an empty building, whose fireplaces and inside walls were in a neglected state, and were found to contain many sandflies. This building is situated about 70 yards away from the more heavily affected barrack room. Steps were taken to have this building overhauled, and necessary repairs carried out, and resulted in a very definite improvement.

Treatment of breeding places with a larvicidal solution.

Reports by other workers on the treatment of breeding places with larvicides indicate varying degrees of success. Puri tested thirteen different preparations on artificially made breeding places at Landi Kotal and Karnal, and found that carbon bisulphide emulsion was the only one which proved effective in every case. Mehta (1935) reports favourably on the use of carbon bisulphide emulsion, diluted 1/200 with water and sprayed on likely breeding places in the amount of one quart per square foot. Smith, Mukerjee and Lal (1936) sprayed proved breeding places of *P. argentipes* with two larvicides, i.e., (*i*) Necrosene, and (*ii*) a crude oil and kerosene mixture. In the laboratory the results were very satisfactory, but when sprayed on proved breeding sites in Nature and the surrounding areas, the solutions were less effective, and the numbers of sandflies in neighbouring houses and cattle sheds were unaltered.

As a disinfectant agent, on this occasion, it was decided to use a solution of naphthalene, which has a high insecticidal power. Besides its familiar domestic use against moths, it was used extensively in the Great War (in the form of N. C. I. powder) against louse infestation. McKenny Hughes (1937) reports successful results when this substance (in the form of heavy naphtha) is used against bed bugs.

Preliminary experiments were made with various solvents. At room temperature, naphthalene was found to be soluble as follows: methylated spirit 6 per cent; absolute alcohol 8 per cent; kerosene 15 per cent; petrol 20 per cent; carbon tetrachloride 40 per cent. After heating to 75°C., its solubility in methylated spirit and carbon tetrachloride was 50 per cent and 70 per cent respectively. Efforts were made to increase the quantity of naphthalene dissolved by making up saturated solutions in kerosene, at room temperature, and adding 1/10th the volume of a concentrated solution of naphthalene in carbon tetrachloride, at 75°C. This was unsuccessful as the excess naphthalene crystallized out on cooling. It was finally decided to use a concentrated solution of naphthalene in kerosene as a basis, with the addition of small quantities. of petrol and carbon tetrachloride. The former of these was expected to give increased volatility and power of penetration into small cracks, while the latter should, in some degree, decrease the inflammability. In addition, both substances were expected to increase the percentage of naphthalene dissolved.

The conditions required for a suitable venue for this type of work are a well-isolated building, at least 100 yards from any other occupied premises, with a large

sandfly population, and a fairly high incidence of the fever among the inmates. After a search, a place which seemed to produce these criteria was found in the British Infantry Barracks at Nowshera Cantonment, 28 miles from Peshawar, consisting of a medium-sized double-story building, occupied by a detachment, about 100 strong, of the 1st Dorsetshire Regiment. This building occupies three sides of a square, each side of which is about 80 yards, and the nearest occupied premises were, at the time, about 150 yards away. The surrounding terrain is bare and stony and appeared unlikely to provide an excessively large number of breeding places for sandflies. This building was used as a test area and another barrack, about one and a half miles away, occupied by the 7th Field Battery, R.A., was selected as a control.

A squad of six B. O. Rs. from the Dorsetshire Regiment, to act as sandfly collectors, was placed at the disposal of the Inquiry and, after preliminary training, both the test and control areas were surveyed. The larvicidal solution was made up in four gallon drums, each of which contained the following :—

				lb.
Kerosene, 3rd grade	..	..	..	35
Naphthalene, powdered	..	..	..	5
Petrol ($\frac{1}{4}$ gallon)	..	..	..	$2\frac{1}{2}$
Carbon tetrachloride	..	..	..	$\frac{1}{2}$

This solution contained rather more than 13 per cent of naphthalene.

The treatment of the test area was carried out on 1st August. Two men were detailed to go over the interior of the main building and also various small buildings in the neighbourhood, used as store rooms, bicycle sheds, etc., and to spray all cracks and interstices on the inside walls very thoroughly with hand sprays, filled with the solution. On the ground outside a perimeter was marked out at a distance of 120 yards from the main building, to allow a liberal margin over the theoretical range of flight of *P. papatasii*, which is believed to be between 50 and 80 yards. All likely breeding places within this perimeter were treated with solution, by pouring it on freely, so as to saturate the ground. Particular attention was paid to places where the soil was loose, to the sides of culverts and drains, and to the sides of houses, especially at the junction of wall and ground. The amount of solution thus used was 32 gallons, this being all that had been made up.

Six weeks later, on 15th September, this treatment was repeated, using 40 gallons of a plain solution of naphthalene in kerosene, that had been allowed to stand for three days (it having been noted that, at room temperature, naphthalene is fairly slowly soluble in kerosene). The strength of this solution was about 12 per cent. It probably would have been preferable to carry out this second treatment after an interval of four to five weeks, but this was prevented by the demands of other portions of the Inquiry.

Periodic surveys were performed on both test and control areas, and the numbers of sandflies caught are shown in Table II; in the final column of this table, the figure for the test area is expressed as a percentage of the nearest control area figure.

TABLE II.

Numbers of sandflies caught in test and control areas, during period of observation.

Date.	Test area.	Control area.	Test as percentage control.
31–7–38 ..	31	..	42

1st application of solution on 1–8–38.

Date.	Test area.	Control area.	Test as percentage control.
2–8–38 ..	..	75	..
24–8–38 ..	43	..	63
26–8–38 ..	..	68	..
9–9–38 ..	90	..	42
10–9–38 ..	106	..	48
13–9–38 ..	..	219	..
14–9–38 ..	199	..	90

2nd application of solution on 15–9–38.

Date.	Test area.	Control area.	Test as percentage control.
20–9–38 ..	..	316	..
21–9–38 ..	139	..	44
28–9–38 ..	98	..	48
30–9–38 ..	..	209	..
3–10–38 ..	101	..	50
5–10–38 ..	..	203	..

The weather during almost all this period was hot and dry. There were two wet days on 29th and 30th August and some rain on the morning of 21st September.

The figures in this table show no evidence of a decrease in the number of flies in the test area, as compared with the control area. Column 4 shows that, with one exception, the test area figure, regarded as a percentage of the control, varied very little.

The figures for the incidence of the fever in both areas were obtained, and are summarized in Table III :—

<div align="center">TABLE III.</div>

Showing the average number of hospital admissions for sandfly fever, per 1,000 men, each week.

Period.	Test unit.	Control unit.	Whole station.
Pre-observation (18th April to 31st July).	2·44	11·75	4·46
Observation (1st August to 5th October).	0·44	4·22	1·44
Post-observation (6th October to 11th November).	1·25	1·60	1·33

These figures provide no evidence of a reduction in the incidence of the fever in the test unit, as a result of the treatment of the soil, particularly when it is stated that the detailed figures for the weekly admission rate in Nowshera, and for most stations in the Peshawar district in 1938, showed a large number of cases from mid-April onwards, up to a peak point about the end of June. Following this was a gradual decline until early in October, succeeded by a small secondary rise during the next four or five weeks. This small secondary rise towards the end of the hot weather has also been noted in the incidence tables of other years. It may be connected with the return to the plains of numbers of troops, who, having spent several months in the hills, are less 'salted' against the disease, and also less well versed in the various protective measures. This view is supported by the fact that three cases of the fever occurred in the 'test' unit of the present investigation, between the 9th October and 7th November. Each of the three individuals concerned had returned to the plains on 5th October after a period of four to five months in the hills, at Cherat.

Simultaneously with this work in Nowshera, the larvicidal power of the naphthalene in kerosene solution was tested against some 'laboratory-bred sandflies. The solution was applied to artificial breeding places, by two methods, viz. :—

(a) Spraying from a hand spray.

(b) Pouring it on so as to saturate the soil.

In both cases, no adult flies emerged from the treated breeding jars, but from an untreated control, adults emerged. This test was carried out in duplicate, with identical results. In one set of breeding jars, the fed females had been introduced five weeks previously, and presumably the pupal stage had been reached by the time the solution was applied. It would thus appear that the solution is not only larvicidal, but will destroy breeding sandflies at their most resistant stage (pupation).

In view of this, the failure of the experiment is attributed, not to the use of an unsuitable solution, but to the extreme difficulty of applying it to every breeding

place in the area. It also drew attention to the heavy financial effect of this type of work. The solution in its simplest form costs about Re. 1 per gallon and 40 gallons appear to be insufficient for one treatment of the ground around a single building, on the bare and dried up terrain of Nowshera.

Insecticidal work with 'Lethane', distributed in a 'Phantomyst' Atomizer.

Towards the end of the season, an opportunity occurred of carrying out some spraying of buildings, as an anti-sandfly measure. The solution employed consisted of 5 per cent 'Lethane', in 2nd grade kerosene, used in a 'Phantomyst' Atomizer Type D. This apparatus is the property of Peshawar District Headquarters and was placed at the disposal of the Inquiry for these tests. 'Lethane (384)' is an aliphatic thiocyanate, manufactured by the firm of Charles Lenning & Co.; it is distributed in India by Messrs. William Jacks, Karachi. Strother Smith (1938) reports it as being effective, in 3 per cent solution, against mosquitoes and house flies.

Suitable barrack rooms, of about 3,000 cubic feet interior capacity, were selected and their sandfly population surveyed by a squad of catchers. Spraying was then carried out for one hour (from 5 p.m. to 6 p.m.), during which time the doors and windows were kept closed. Later that evening, from 8 p.m. to 9 p.m., and again the following morning, from 6-30 a.m. to 7-30 a.m., surveys were carried out in the sprayed rooms, and also in unsprayed controls. A consideration of the numbers of flies caught at these surveys suggested that this form of treatment entirely eradicates all sandflies from a room for some hours, and causes a substantial reduction throughout the ensuing night.

Table IV gives an example of the results obtained :—

TABLE IV.

Numbers of sandflies caught in No. 6 Block, Connaught Lines, Peshawar.

	Date.	Time.	Test room.	Control room.
Before spraying .. {	21-10-38	a.m.	29	28
	..	p.m.	30	30
After spraying .. {	24-10-38	p.m.	*Nil*	15
	25-10-38	a.m.	3	28

During the spraying, live sandflies were placed in muslin cages near the Atomizer, and were killed outright, so that the solution would appear to be actively insecticidal, and not merely repellent. It is possible, however, that the concentration of Lethane inside these cages may have been higher than in the rest of the room.

As far as could be ascertained, none of the personnel sleeping in the treated room suffered any reaction from the presence of traces of Lethane in the atmosphere. Further tests with this apparatus were curtailed, owing to the somewhat abrupt termination of the sandfly season.

SUMMARY.

1. An observation of the epidemiology of sandfly fever among Indian troops in Peshawar suggests that the hill races, and persons coming from outside the ' sandfly areas ', are more susceptible to the disease.

2. The time required for metamorphosis in laboratory-bred *P. papatasii* varies considerably with changes in atmospheric temperature.

3. *P. papatasii* appear capable of flying up to 70 feet above ground, and no evidence could be found that persons sleeping on upper stories enjoyed any protection against their bites.

4. The minimum wind velocity against which *P. papatasii* cannot fly appears to be in the region of 1·8 miles per hour.

5. A solution of naphthalene in kerosene, actively larvicidal to laboratory-bred sandflies, when applied to as many potential breeding places as possible within 120 yards of an isolated building, failed to effect any material reduction in the sandfly population.

6. A 5 per cent solution of ' Lethane (384) ', used in a ' Phantomyst ' Atomizer, appeared to be efficient in eliminating *P. papatasii* from a barrack room for more than 12 hours. The ' Lethane ' vapour appears to be actively insecticidal against *P. papatasii*.

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CHEMOTHERAPY OF FILARIAL INFECTION.

BY

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FILARIASIS is one of the most important tropical diseases distributed widely in all parts of India causing considerable suffering and even death by acute inflammatory attacks. The infection is caused by two species of parasites, viz., *Wuchereria bancrofti* and the adult of the *Microfilaria malayi* transmitted chiefly by the *Culex fatigans* and *Mansonioides annulifera* respectively. Both are parasites of the lymphatic system in man. The former infection is more widely spread and is mainly prevalent along the coastal regions and low-lying areas near big rivers. A survey of the distribution reveals that Bengal is the most heavily infected province ; Bihar, the United Provinces, Orissa, and Madras coming next in order. Of the other provinces, the Central Provinces and Nizam's Dominions have isolated areas showing the infection, while the Punjab, the North-West Frontier Province, and Rajputana are practically free. Filarial infection ultimately leads to lymphangitis, adenitis, abscess, lymph-varix, hydrocele, chylocele, chyluria, and elephantiasis of the extremities and genitals by causing obstruction and damage to the lymphatic vessels and glands.

Considering the wide prevalence of the infection and unsatisfactory methods of prevention, the treatment of filariasis by chemotherapeutic methods becomes an important problem. In 1929, we reviewed the existing knowledge with regard to the treatment of filariasis and concluded that of the number of drugs that had been tried up to that time none had given satisfactory results. It may be pointed out here that the treatment should be directed against the adult parasites living in the lymphatics, as well as against the embryos which are in the blood as the former bring about gradual obstruction of the lymphatic system and the latter disseminate infection to others through the agency of the mosquitoes. The embryos are discharged by the adult worm into the lymphatic vessels whence they are carried to the blood stream. In the blood the microfilariæ exhibit a nocturnal periodicity. With the onset of filarial disease the embryos are shut out from the

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blood circulation on account of lymphatic obstruction except in early cases of chyluria. The obstruction in chyluria patients is at the juxta-aortic glands where free anastomosis between the glands and the cysterna chyli enables the embryos to reach the blood stream. It is important that the treatment for the infection should be carried out in the early stages before any great damage is done to the lymphatics. At this stage, the number of embryos per unit of blood gives a satisfactory idea of the influence of a drug on the adult parasite.

We have carried out a systematic study of the chemotherapeutics of filarial infection at the School of Tropical Medicine, Calcutta, for the last 10 years. Patients in various stages of infection were treated at the Filariasis Clinic of the School by drugs whose therapeutic efficacy in other parasitic diseases was well known. Suitable cases were admitted into the Carmichael Hospital for Tropical Diseases for observation and drugs were studied exhaustively for their filaricidal properties. Patients admitted into the hospital for infections other than filariasis in whom filarial infection was found subsequently, were watched for the effect, if any, of drugs administered for the primary infection on the filarial infection. Cases of malaria, kala-azar, dysentery, hookworm infection, etc., who in the course of routine investigation in the hospital showed filarial infection have in this way been studied and the results are discussed in this paper.

Chemotherapeutic treatment of filariasis has not received as much attention as it deserves. The literature up to 1929 was reviewed in our previous paper (Chopra and Rao, 1929) and we discussed the results obtained with a number of organic compounds containing antimony, arsenic and mercury, and other synthetic preparations. Since then, Bose (1930) recorded his observations on antimony, arsenic, mercury, and various synthetic compounds. Phelps and his colleagues (1931) tried intramuscular injections of chenopodium oil with encouraging results. Sherwani (1932) obtained satisfactory results with neostibosan in a case of chyluria. Ashford and Synder (1933) observed that gentian violet orally caused a diminution of microfilariæ in 11 out of 13 cases in Porto Rico. van Slype (1933) in his contribution on gold therapy of filariasis observed that there was a marked diminution and even disappearance of embryos as long as the treatment lasted but they re-appeared after it ceased. Menon (1930) had disappointing results with sulpharsenamide. Prontosil and trypaflavine (1937) have been tried without effect. Schmidt and Peter (1938) in their book on antimony compounds observed that fouadin is a specific in canine filariasis. We have during the last several years tested a number of drugs *in vitro* and *in vivo* in filarial infection and the results obtained form the subject of this paper.

Studies on Microfilaricidal Substances *in vitro*.

The effect of drugs on the life and activity of microfilariæ *in vitro* may not be the same as *in vivo*, yet it gives some indication of the therapeutic efficacy of the drug as a guide for its use in human infection. It is well known that microfilariæ in this infection appear in the peripheral blood during night and can be drawn out along with the blood in citrate solution for the purpose of examination. One c.c. of blood so drawn in citrate at midnight from a filarial carrier shows a large number of embryos which continue to live actively at body temperature for 24 hours and much longer at room temperature of 75°F. The blood so diluted is drawn by capillary pipette and a few drops taken on hollow ground slides are examined under

the low power ($\frac{2}{3}$ objective). Such a preparation generally shows at least 10 to 15 embryos for observation. It is, however, found that the microfilariæ cluster together in the test-tube and it is difficult to get an even distribution of them in the solution. In addition to this, since the blood has to be drawn at midnight and kept for over 12 hours before tests are carried out, the embryos are generally found to be not so active as in freshly drawn blood. The embryos in blood collected at midnight are, therefore, unsatisfactory for the purpose of these tests. We found that in hydrocele fluid the state of affairs for studying the filaricidal properties of drugs was ideal. Microfilariæ are present in filarial hydrocele fluids at all times and the fluid can be aspirated at any time it is required and observations can be made immediately. The embryos are fairly evenly distributed in the fluid. They are found to live for 48 hours at room temperature and their activity is not decreased within this period as in the case with those obtained from peripheral blood. The technique for observing the action of the drug on the embryos is as follows : The drug to be tested is prepared in various dilutions generally 1 in 100, 1 in 1,000, 1 in 5,000, and 1 in 10,000 in distilled water. Glass-slides with a circular depression to hold the liquid are taken and in each of them equal volumes (about 0·1 c.c. to 0·2 c.c.) of freshly drawn hydrocele fluid and of the drug dilution are mixed. The slide is covered with a cover-glass and sealed with vaseline to prevent evaporation. The movements of the embryos are watched under a ($\frac{2}{3}$ objective) every few minutes. Some drugs even in concentrations of 1 in 100 have no adverse effect on the embryos. These observations are carried out generally for two hours or longer and the results recorded. Some of the drugs are so strongly larvicidal that they kill the embryos in dilution of 1 in 10,000 in 10 seconds ; this is the case with atebrin, plasmochin and some others. The actual results obtained with the various drugs are summarized in the following table :—

TABLE I.

Showing the effect of the drug in different dilutions on microfilaria in vitro.
A = active ; S = sluggish ; D = slow death ; K = killed immediately.

Drug.	DILUTION.				Remarks.
	1/10,000	1/5,000	1/1,000	1/100	
Fouadin	A	A	A	A	Sluggish movement after 1 hour.
693 (Neostibosan)	A	A	A	A	,, ,, ,, ,,
Sdt. 561 (Bayer)	A	A	A	A	,, ,, ,, ,,
Tristibine (Meurice)	D	D	D	K	5 to 15 minutes. ,, ,,
Stibilase (Meurice)	D	D	D	K	,, ,,
'A 534' (P. D. & Co.)	A	A	A	A	
Anthiomaline (M. & B.)	A	A	A	A	
Soamin	A	A	A	A	1 hour.
N. A. B.	A	A	A	A	,,
Sulfarsenol	A	A	A	A	,,
Arsiminol	A	A	A	S	,,
Arsylene 'Roche'	A	A	A	A	,,
Carbarsone (Lilly)	A	A	A	A	
Stovarsol (M. & B.)	A	A	A	A	
Cuprochin (Meurice)	A	A	S	K	20 minutes.
Cuprion (Bayer)	A	A	A	A	
Atebrin	D	D	K	K	10 ,,

TABLE I—*concld.*

Drug.	DILUTION.				REMARKS.
	1/10,000	1/5,000	1/1,000	1/100	
Plasmochin	D	D	K	K	10 minutes.
Cilional	A	S	D	D	¼ hour.
Prontosil (Bayer) ..	A	A	A	A	
Soluseptasene (M. & B.) ..	A	A	A	A	
Rivanol	A	A	S	D	
Trypaflavine ..	A	A	S	D	
Cobra venom ..	A	A	S	D	15 minutes = S, ½ hour = D.
Russell's viper venom ..	A	A	A	A	

None of the other organo-metallic compounds shown in Table II has any effect on the microfilariæ *in vitro* in any of the dilutions shown above.

A perusal of Table I shows that several compounds have high filaricidal properties *in vitro*. Acriflavine, trypaflavine, atebrin, plasmochin, trystibine, stibilase, oxyquinoline derivatives, and gentian violet, destroy the embryos in dilutions of 1 in 10,000 in 2 to 5 minutes and in dilutions of 1 in 100,000 in half an hour. On the other hand, several other drugs have absolutely no effect on the embryos even in concentrations of 1 in 100.

It should be noted, however, that *in vivo* we cannot give very high doses of many drugs orally or parenterally without producing harmful effects. Most of the drugs administered in therapeutic doses occur in concentrations of only 1 in 200,000 to 1 in 100,000 in the blood. In these dilutions the embryos can live *in vitro* unaffected for more than two hours. It should be noted that the above concentrations are the maximum which are attained in the blood when the drugs are administered and that even these low concentrations do not generally last for more than two hours (Chopra and Roy, 1934).

Atebrin even in very high doses *in vivo* is found to have no effect on the microfilariæ or on the adult worm. Fouadin and other antimony compounds are unable to kill the embryos *in vitro* even in high concentrations but *in vivo* the embryo-count decreases appreciably after a few doses.

It will be seen from the above that the studies of drugs *in vitro* give generally no clue to the antifilarial properties of the drug *in vivo*, as some drugs such as plasmochin and acriflavine which are lethal to the parasite *in vitro* have no effect *in vivo*, while other drugs, which show no lethal effect *in vitro*, produce appreciable reduction of the embryos *in vivo*.

CLINICAL TRIALS OF VARIOUS DRUGS IN FILARIAL INFECTIONS.

Patients at different stages of filarial infection were treated at the Carmichael Hospital for Tropical Diseases with a variety of drugs including some which had been reported by different observers to have therapeutic value. The histories of all patients were recorded carefully and observations made on the microfilaria counts before, during, and after treatment. The general and local reactions with the drugs tested were noted. The results of the trials are summarized in Table II :—

TABLE II.

Drugs.	Number of patients treated.	Dose.	Total amount of drug administered.	Route.	Interval.	Reaction.	Results.
Antimony Compounds.							
1. Fouadin (Bayer) ..	14	1·5 c.c.—5 c.c.	80 c.c.	I. V.	Alternate days	Gastritis, enteritis.	Temporary reduction of M. f.
2. Neostibosan (Bayer) ..	10	0·1 g.—0·3 g.	3 g.	"	Daily	..	do.
3. Sdt. 561 (Bayer) ..	4	6 c.c.—9 c.c.	87 c.c.	I. M.	"	..	do.
4. Trystibine (Meurice, Belge)	6	0·1 g.	1 g.	I. V.	Twice weekly	Fever, pain joints.	do.
5. Stibilase (Meurice, Belge) ..	6	0·1 g.	1 g.	"	"	do.	do.
6. 'A 534' (P. D. & Co.)	5	0·1 g.	1 g.	I. M.	"	Pain at site of injection.	No effect.
7. Anthiomaline (M. & B.) ..	7	3 c.c.—5 c.c.	30 c.c.	"	"	do.	Slight reduction in M. f. temporarily.
Arsenic Compounds.							
8. Soamin (B. W. & Co.) ..	28	2 grains.	20 grains.	Subcutaneous.	Alternate days	..	No effect on M. f.
9. N. A. B. (M. & B.) ..	9	0·3 g.—0·6 g.	2 g.	I. V.	Once weekly	..	do.
10. Sulfarsenol (A. F. D.) ..	12	18 ctg.—36 ctg.	2 g.	"	"	..	do.
11. Arsiminol ..	2	3 c.c.	12 c.c.	I. M.	Twice weekly	Fever, pain loins.	do.
12. Arsylene 'Roche' ..	15	2 c.c.	24 c.c.	"	"	..	do.
13. Sulpharsphenamine ..	4	0·3 g.—0·4 g.	1·4 g.	I. V.	Once weekly	Fever	do.
14. Carbarsone ..	20	0·25 g.	5 g.	Oral	Daily	..	do.
15. Stovarsol ..	5	4 grains	80 grains	"	"	..	do.

TABLE II—*contd.*

Drugs.	Number of patients treated.	Dose.	Total amount of drug administered.	Route.	Interval.	Reaction.	Results.
Gold Compounds.							
16. Crisalbine (M. & B.) ..	2	0·15 g.	1·5 g.	I. V.	Twice weekly	Fever	No effect on M. f.
17. Solganal B. Oleosum (Schering).	12	0·1 g.	3–6 g.	I. M.	,, ,,	..	do.
Mercury Compounds.							
18. Salyrgan (Bayer) ..	5	2 c.c.	20 c.c.	I. M.	Alternate days	..	No effect on M. f.
19. Novasurol (Bayer) ..	4	2 c.c.	20 c.c.	,,	,, ,,	..	do.
Copper Compounds.							
20. Cuprochin (Meurice, Belge)	6	0·2 g.	100 grains	Oral and I. V.	Daily and twice weekly.	..	No effect on M. f.
21. Cuprion (Bayer) ..	10	0·01 g.	0·15 g.	I. M.	Twice weekly	Pain at site of injection.	do.
22. Sdt. '242' Copper in oil (Bayer).	3	2 c.c.	10 c.c.	,,	,, ,,	do.	do.
Zinc Compounds.							
23. Sdt. '409' (Bayer) ..	2	0·2 grain	2 grains	I. M.	,, ,,	Pain at site of injection.	No effect on M. f.
24. Sdt. '322' (Bayer) ..	2	0·2 ,,	2 ,,	,,	,, ,,	do.	do.
Tin Compounds.							
25. Tin complex salt (Bayer) ..	2	0·2 grain	2 grains	I. M.	,, ,,	Pain at site of injection.	No effect on M. f.

						Pain at site of injection.	No effect on M. f.
Lead Compounds.							
26. Sdt. '302' (Bayer) ..	2	0·2 grain	2 grains	I. M.	Twice weekly	Pain at site of injection.	No effect on M. f.
Bismuth Compounds.							
27. Bivatol (A. F. D.) ..	3	1 c.c.	10 c.c.	I. M.	Twice weekly	Pain at site of injection.	No effect on M. f.
28. Bismostab (Boots) ..	5	1 c.c.—2 c.c.	10 c.c.	,,	,,	do.	do.
29. Troken Amp. (Bayer) ..	2	0·2 grain	2 grains	,,	,,	do.	do.
30. S. W. 277 (Bayer) ..	2	0·2 ,,	2 ,,	,,	,,	do.	do.
Iodine Compounds.							
31. Yatren (Bayer) ..	6	4 grains	500 grains	Oral	Daily	..	No effect on M. f.
32. Abrodil (Bayer) ..	5	19 c.c.	10 c.c.	I. V.	Once	..	do.
33. Per-abrodil (Bayer) ..	4	19 c.c.	10 c.c.	,,	,,	..	do.
34. Uroselectan B. (Schering)	6	20 c.c.	20 c.c.	,,	,,	..	do.
35. Pot. Iodide ..	10	10 grains	300 grains	Oral	Daily	..	do.
Synthetic Products.							
36. Atebrin (Bayer) ..	6	0·1 g.	1·5 g.	Oral	Daily	..	No effect on M. f.
37. Atebrin Musonate (Bayer)	5	0·2 g.	1 g.	I. M.	,,	..	do.
38. Tebetrin (Howard) ..	4	0·1 g.	1 g.	Oral	,,	..	do.
39. Malarcan	5	0·1 g.	1 g.	,,	,,	..	do.
40. Plasmochin (Bayer) ..	4	0·025 g.	0·25 g.	,,	,,	..	do.
41. Plasmochin Simplex (Bayer)	4	0·01 g.	10 c.c.	I. V.	,,	..	do.

TABLE II—*concld.*

Drugs.	Number of patients treated.	Dose.	Total amount of drug administered.	Route.	Interval.	Reaction.	Results.
42. Gametoxan. (Sfeat) ..	6	0·1 g.	1 g.	Oral	Daily	..	No effect on M. f.
43. Cilional (Bayer) ..	6	0·05 g.	1·10 g.	"	"	..	do.
44. 'A 539' (P. D. & Co.) ..	10	0·1 g.	1 g.	I. V.	Twice weekly	..	do.
45. Soluseptasene (M. & B.) ..	5	10 c.c.	100 c.c.	I. M.	Daily	..	do.
46. Proseptasene (M. & B.) ..	12	0·5 g.	15 g.	Oral	"	..	do.
47. Prontosil (Bayer) ..	18	0·5 g.	15 g.	"	"	..	do.
48. Entero-Vioform (Ciba) ..	6	0·025 g.	0·75 g.	"	"	..	do.
49. Congo-Red	2	1 per cent 5 c.c.	35 c.c.	I. V.	"	..	do.
50. Paludex	6	5 grains	75 grains	Oral	"	..	do.
51. Quino-paludex ..	6	5 "	75 "	"	"	..	do.
52. Gentian violet ..	2	2 per cent 2-5 c.c.	20 c.c.	I. M.	Twice weekly	Pain at site of injection.	do.
53. Rivanol	2	5 per cent 1-5 c.c.	20 c.c.	"	" "	do.	do.
54. Trypaflavine ..	4	3 per cent 2-5 c.c.	35 c.c.	I. V.	" "	..	do.
55. Surfen (Bayer) ..	6	1 per cent 2 c.c.	10 c.c.	I. M.	" "	Pain at site of injection.	do.
56. Atophan (Schering) ..	2	7½ grains	90 grains	Oral	Daily	..	do.
57. Caprokol	5	0·15 g.	3·75 g.	"	"	..	do.
58. Sod. mandalate ..	4	4 days' course	..	"	"	..	do.

No.	Drug	Dose	Dose	Route	Frequency		M. f. count
59.	CCl₄	1 c.c.	..	"	"	..	do.
60.	C₂Cl₄	1 c.c.	..	"	"	..	do.

Vegetable Drugs.

No.	Drug	Dose	Dose	Route	Frequency		M. f. count
61.	Oil Chenopodium	0·5—1 c.c.	10–15 c.c.	I. M.	Twice weekly	Pain at site of injection.	Slight reduction in M. f. count.
62.	Quinine bi-hydrochloride	10 grains	70 grains	"	Once daily	do.	No variation in M. f. count.
63.	Quinine and acid salicylic	10 "	50 "	"	" "	do.	do.
64.	Cinchona febrifuge	15 "	150 "	Oral	Daily	do.	do
65.	Kurchi Extract Liquid	1 dram	8 oz.	"	"	..	do.
66.	Caesalpina bonducella	4 nuts	once	"	"	..	do.
67.	Extract Lodh Liquid	1 dram	12 oz.	"	"	..	do.
68.	Emetine hydroch.	1 grain	6–12 grains	I. M.	"	Pain at site of injection.	do.
69.	Berberine sulphate	0·1 g.	1 g.	"	Twice weekly	do.	do.
70.	Thymol	3 grains	42 grains	Oral	Daily	..	do.
71.	Santonin	2 "	6 "	"	"	..	do.
72.	Oil Hydnocarpus	5 c.c.	100 c.c.	I. M.	Twice weekly	..	do.
73.	Cobra venom	5 mouse units	500 mouse units	Hypo.	"	..	do.
74.	Viper venom	5 " "	200 mouse units	"	"	..	do.
75.	Human malaria (induced)	..	..	..	..	..	M. f. count temporarily reduced during fever.
76.	Monkey malaria (induced)	..	..	..	..	..	do.

The present investigations were carried out with a view to find a drug lethal to filarial parasite. Temporary relief, decrease in the duration of the attacks of lymphangitis, temporary decrease in the number of microfilariæ are not indications of the therapeutic value of the drug. It has to be noted that filarial infection by itself exhibits wide variations; several instances are known where persons with microfilariæ in blood remain free from lymphatic obstruction for several years. In some other, attacks of lymphangitis recur periodically at frequent intervals and the attacks decrease and disappear without treatment. Several patients have stated that their attacks at times occur at close intervals and later only at long intervals. This natural variation in filarial infection often misleads the patient and the physician and hence one hears unwarranted claims for several drugs as antifilarial specifics. With a view to study accurately the chemotherapeutic activity of the drugs in this infection we have administered them to patients and have recorded over a long period the effects which were produced. Thus, by studying a number of cases in each series of experiments we were able to eliminate the variations in the microfilaria counts normally due to the parasite.

The drugs selected for investigation covered a wide range. Most of them were chosen because of the reports in the literature as to their beneficial effects in allied parasitic infections. Some compounds were especially prepared by well-known pharmaceutical manufacturers including Bayer, May & Baker, Parke, Davis & Co., etc. Several drugs were included in our studies on account of their curative effects claimed by the patients attending the out-door department. A few of the indigenous drugs were also included in the list. These were kindly prepared by Dr. S. Ghosh, Professor of Chemistry at the Calcutta School of Tropical Medicine.

The results shown in Table II are briefly discussed below :—

Antimony compounds.—Practically all available organic compounds containing antimony were investigated. Of these Fouadin and Anthiomaline contain antimony in trivalent form; the remaining compounds contain the metal in pentavalent form.

Trystibine and stibilase were highly toxic, and could not be tolerated in doses higher than 0·1 g. These could be given only intravenously. Sdt. 561 (Bayer) is put up in clear solution in ampoules ready for use having 26 per cent antimony in pentavalent form and is quite non-toxic. The drug could be administered intramuscularly daily without causing reaction. Anthiomaline and ' 534 ' can only be given intramuscularly and both of them cause severe reaction with pain and swelling at the site of the injection. Fouadin gave the most satisfactory result in filarial infection. The drug is put up in solution in rubber-capped phials ready for use and can be administered subcutaneously, intramuscularly, or intravenously. It is non-toxic. The effect of the drug on the filarial parasite appears to be only temporary as the microfilariæ re-appear in the blood after a lapse of a few days. This drug is very useful in controlling the inflammation and fever in filarial patients for a comparatively long period of time.

In a few cases of chyluria the urine cleared up completely even after one dose of this drug.

Arsenic compounds.—Of the arseno-compounds investigated, two of them, soamin and arsylene, have arsenic in trivalent form. The former is a popular brand of arsenic compound put up in tablet form by Burroughs Wellcome & Co. It can

be given subcutaneously, intramuscularly or intravenously. The practice followed at the School is to give a total of 20 grains of this drug in divided doses of 2 grains administered subcutaneously on alternate days. The drug is generally non-toxic. We have, however, found that on very rare occasions patients, particularly the very young and the aged, may exhibit toxic symptoms even after the first injection. It is, therefore, not advisable to administer it to such susceptible individuals. The belief entertained by some practitioners that the drug causes optic atrophy appears to be without foundation. In a very large number of patients treated by us at the School with this drug not a single case of such untoward result has been observed so far. Although there does not appear to be any appreciable diminution in the number of microfilariæ even after a full course, the clinical effects obtained with this drug are very satisfactory. In some cases the patients have remained free from fever and inflammatory attacks for several years after one course of soamin. As compared with other antifilarial drugs soamin is cheap and can be administered easily. It is, therefore, the most suitable among the arseno-compounds for treatment of filariasis.

Arsylene 'Roche' was formerly marketed in ampoules and was described by the manufacturers as equivalent to soamin. The drug has no effect on the parasite. Arsiminol is highly toxic. The remaining compounds novarsenobillon, sulfarsenol, sulpharsphenamine, etc., are well-known preparations and are as satisfactory as soamin, but are much more costly. Of these sulpharsphenamine is comparatively more toxic.

Carbarsone and stovarsol given orally are effective in the treatment of amœbic infection. They are comparatively non-toxic. They do not have any effect on the parasite or produce any beneficial effect clinically.

In general, all the above arsenic compounds have been found to improve the general health of the patients.

Copper compounds.—The administration of copper containing organic compounds was taken up on account of the references in Ayurvedic literature as to the efficacy of this metal in the treatment of filariasis. Cuprion and Sdt. '242' were especially prepared by Bayer for this investigation. The former is an organic compound in powder form and is administered diluted with sterile distilled water intramuscularly. Sdt. '242' is a compound containing the metal in oil, and is also given intramuscularly. Cuprochin prepared by Meurice (Belge) was supplied both in tablet form for oral use and also for intravenous administration. Of these cuprion was very toxic. None of these compounds gave any positive result with regard to the effect on the parasite. An improvement in general health of the patients was noticed with all these compounds.

Other organo-metallic compounds.—These included organic compounds containing gold, bismuth, mercury, zinc, tin, and lead. Gold has been reported as having antifilarial properties. This claim, however, has not been supported by our investigations. Both crisalbine and solganal B. oleosum did not produce any diminution of microfilariæ even after a full course of 1 g. of the former and 6 g. of the latter. As may be expected with these compounds a general tonic effect was observed.

The results obtained with bismuth compounds were similar to those obtained with several of the arsenic and antimony compounds. No appreciable diminution in the microfilariæ was noticed with any of them. Similar negative results were obtained with the organic compounds containing mercury, zinc, tin, and lead. In one case severe lead poisoning resulted after the administration of 0·5 g. of Sdt. ' 302 ' (lead compound).

Iodine compounds.—It was reported by Wood (1929) that one injection of sodium iodide compound given for pyelography had cured chyluria. A careful investigation of a number of iodine compounds was taken up but the results were not satisfactory. Abrodil, perabrodil, and uroselectan B are generally used for pyelography. In susceptible individuals iodism may appear.

Synthetic products.—The synthetic products may be broadly classified under three groups : (1) compounds of the atebrin-plasmochin type which are used for malarial infection, (2) compounds of the prontosil type which are used for the treatment of coccal infections, and (3) acriflavine compounds. Numbers 36 to 43 in Table II belong to the first group, numbers 45 to 47 to the second group, and numbers 53 to 55 to the third group. None of the drugs belonging to the first group such as atebrin, plasmochin, tebetrin, malarcan was found to show any effect on the filarial infection although the embryos were killed *in vitro* almost immediately with fairly large concentrations. Atebrin and its derivatives were given orally and by intramuscular injections. Tebetrin which is marketed by Howards is stated to be equivalent to atebrin. Plasmochin was used both orally and by intravenous injections. In some cases there was toxic reaction. Cilional is a later preparation marketed by Bayer as a substitute for plasmochin and is less toxic.

Prontosil has been successfully used in recent years in controlling streptococcal and allied infections. It is a sulfanilamide administered orally and intramuscularly, marketed by Bayer in the form of tablets and as solution for injections. Various similar compounds are also marketed by other manufacturers. Prontosil has been tried on a large number of patients and does not appear to have any effect on the parasite. It is, however, of great value in the treatment of secondary infections which are common in filarial patients, especially in advanced stages of lymphatic obstruction. The same thing applies to the other drugs of this type.

Of the acriflavine compounds trypaflavine has been reported (Santos, 1936) to give beneficial results in filarial infection. Careful investigation of this compound on a number of filarial patients, however, did not support these findings. None of these compounds produced any appreciable reduction of the embryos in the blood.

Vegetable drugs.—Quinine compounds were administered orally and by injection, but had no effect on the parasite. The other vegetable drugs were chosen because of their reported efficacy in allied helminthic infections. Phelps *et al.* (*loc. cit.*) reported that chenopodium oil given intramuscularly reduced the number of embryos in the circulation and checked the attacks of lymphangitis. The oil was tried on several patients. The injections produced painful reactions. A temporary diminution of microfilariæ was observed in all the cases after 8 to 10 injections. Further treatment with the oil could not be continued as the patients were unwilling to submit to this treatment. Hydnocarpus oil which is well known

for its efficacy in the treatment of leprosy was tried on some patients. Cases have come under our observation where, during or after treatment for leprosy with hydnocarpus oil, the patients have developed filarial infection. Our results with this oil in filarial infection have been uniformly negative.

Other products.—Sodium mandalate has been reported to be efficacious in *Bacillus coli* infection, and santonin, thymol, CCl_2, and C_2Cl_4 in eradicating intestinal helminths. Results in all our cases were negative. Emetine hydrochloride and kurchi which are used in the treatment of amœbic infection were also found to be of no value in filariasis.

Cobra venom was found *in vitro* to kill the microfilariæ. A total of 700 mouse units of both cobra venom and viper venom was given to patients hypodermically. Results were negative.

EFFECT OF OTHER INFECTIONS ON FILARIAL PARASITE.

We have already stated that filarial infection is capable of co-existing with other infections both bacterial and protozoal. It has been remarked by Wanhill (1906) that the absence of filarial infection in Jamaica, notwithstanding the presence of *Culex fatigans* in swarms, might be due to the high incidence of malaria in the island. We have studied the effects of malaria on filarial parasites both in induced cases of malaria and in natural infections. We have observed several filarial patients having malarial attacks. We have induced human and monkey malaria on filarial patients and observed no effect on the microfilariæ or the adult parasite. Several cases of syphilis with co-existing filarial infection came under our observation. Similarly other infections such as hookworm, kala-azar, leprosy, and amœbiasis, etc., have no effect on the filarial parasite.

As already mentioned in an early part of the paper malarial infection did not appear to affect the filarial parasite. In many cases a small reduction of the microfilariæ was noticed during and after the paroxysm of fever, but the effect was temporary, the microfilariæ reaching the original level after a few days.

SUMMARY AND CONCLUSIONS.

The paper gives the results of investigations with a large number of drugs in the treatment of filarial infection. Of the organo-metallic compounds, soamin appears to be most satisfactory in controlling the infection in the early stages. It does not, however, exert any lethal effect on the parasite. Next to soamin, fouadin appears to be very useful in this respect. It also does not appear to kill the parasite although it sterilizes the parasite temporarily. None of the other organo-metallic compounds is of any effect in filarial infection.

None of the synthetic products investigated gave any positive result. Prontosil and its derivatives are valuable in the treatment of secondary infection in filarial patients.

Oil of chenopodium reduces the microfilariæ count and also the recurring attacks of lymphangitis. The injections are, however, painful. Neither cobra venom nor Russell's viper venom in therapeutic doses has any lethal effect on the filarial parasite.

The above results indicate that so far no drug has been found which has satisfactory antifilarial properties. Since filariasis is an infection whose distribution is limited only to the coastal towns and low-lying areas, and since the carrier mosquito is known, preventive measures at present form the only successful method of dealing with the disease.

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DIFFERENTIATION OF MICROFILARIÆ OF *WUCHERERIA BANCROFTI* AND *FILARIA MALAYI*.*

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MICROFILARIÆ observed in blood of man in India belong to two species, namely, *Wuchereria bancrofti* (Cobbold) and *Filaria malayi* Brug†. Though filarial infection is known to occur in several parts of India the exact distribution of the two infections is not fully known. The epidemiological factors that determine the incidence of the two infections differ to a great extent as was shown by Iyengar (1938). From the academic point of view as well as the practical aspect of disease prevention it is essential that we should know the type of infection occurring in the locality.

The microfilaria of *F. malayi* has been previously described by Brug (1927, 1929), Rodenwaldt (1933), and Feng (1933) from material collected from the

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* The generic position of the species *Filaria malayi* Brug, 1927, cannot be definitely ascertained at present. It appears likely that Brug used the term *Filaria* in a broad sense, as he also called *Wuchereria bancrofti* as *Filaria bancrofti*. It is perhaps doubtful if *F. malayi* would come under the genus *Filaria*, Müller, 1787 (*sens. strict.*). The species is here designated as *Filaria malayi* as was named by Brug, without prejudice to changing its generic name if at a subsequent date the adult forms are found to be different from our present conception of the genus *Filaria* Müller.

† Two other species of blood filariæ previously recorded from India, namely, *Microfilaria actoni* Rao, 1931, and *Filaria powelli* Penel, 1905, have not been included here. *Microfilaria actoni* Rao is now placed as a synonym of *Filaria malayi* Brug (*vide infra*, page 571). As regards *Filaria powelli* Penel, the validity of the species has been called into question by several authorities. Penel's species is based on an extremely meagre description by Powell (1903) of two specimens of microfilariæ found by him in the blood of a policeman in Bombay. The first of the two specimens measured 131μ long and 5·3μ thick. The measurements of the second specimen were not given; it was stated to be 'distinctly shorter' than the first specimen. They were sheathed and had a stumpy tail. No other anatomical characters are known. As stated by Fülleborn (1929), the stumpy tail mentioned by Powell may be a deceptive appearance of the tail-end being folded forwards. The very short length is suggestive of *F. malayi*, but nothing can be said with any degree of certainty. As was pointed out by Sticker, Schüffner and Swellengrebel (1929) the small length observed in two specimens is not sufficient justification for the erection of a new species, since numerous instances are on record of microfilariæ of *Wuchereria bancrofti* having very short lengths. Fülleborn (1929) is also of the same opinion and considers that the erection of this species based on such small evidence as short length, is untenable.

Dutch East-Indies and China. The present article is a comparative study of the two species made from material collected in India.

Technique.

In routine examinations for filarial infection, air-dried thick smears are steeped in a dilute aqueous solution of methylene blue (15 mg. in one litre of water) for one to two hours and the wet smear examined under the microscope. The solution dissolves out the hæmoglobin and stains the microfilariæ and leuco-cytes deep blue.

For making permanent preparations the slide after immersion in water for an hour is treated with freshly made Bles solution (70 per cent alcohol 90 c.c., formalin 7 c.c., and glacial acetic acid 3 c.c.), washed in 70 per cent alcohol, brought down to water, and stained with Delafield's hæmatoxylin. Excess of stain is reduced with acidulated alcohol and the smear counterstained with an alcoholic solution of eosine, cleared in xylol, and mounted in balsam or euparal.

There is often a certain amount of distortion and shrinkage of microfilariæ, especially in the case oi F. malayi, during the drying of blood smears. To get well-stretched worms for study the writer employs the following method of narcotizing the microfilariæ with menthol prior to drying the smear :—

A drop of blood from a carrier is taken on a slide and mixed with a drop of normal saline ; one or two small crystals of menthol are placed in the blood and the slide left in a moist chamber. When all the microfilariæ have been fully narcotized (which usually takes about half an hour) and are in a well-extended condition, the menthol crystals are removed and the blood spread out to form a smear and then quickly dried. The smear is then placed in water to dissolve out the hæmoglobin, fixed with Bles solution, stained with hæmatoxylin, and counterstained with eosine. Microfilariæ obtained by this method are in a better condition for study than those in ordinary thick-smear preparations.

Material preserved in Azur II-formalin was found to be the most suitable for determining the length of microfilariæ and for studying their internal structure. A drop of blood from a good carrier is shaken up with 3 c.c. of a preserving fluid consisting of sodium chloride 0·8 gramme, formalin 4 c.c., Azur II 0·04 gramme, and water 96 c.c. The sediment at the bottom of the fluid is examined 3 to 4 days later*. The different structures that are of diagnostic importance, namely, the excretory cell and pore, the G-cells, the anal pore, and the tail, are seen best in material preserved in Azur II-formalin. This method has an advantage over others inasmuch as the microfilariæ are not exposed to desiccation at any stage.

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* Fülleborn (1924) observed that material (microfilariæ of *Dirofilaria immitis*) preserved in 5 per cent formalin and examined one to four days afterwards gave the nearest approach to the actual length of the microfilaria as judged by measurements obtained from instantaneous photographs of living microfilariæ, and that after four days there occurred a very feeble but progressive reduction in the total length. In the present studies the microfilariæ were measured from 36 hours to 15 days after time of collection and preservation in Azur II-formalin. There was no appreciable reduction in the total length during this period.

For determining the length of microfilariæ and the positions of the fixed points, camera-lucida drawings were made of each microfilaria and the length measured by ' walking ' over the middle line of the drawing with a pair of dividers or with the help of a curvimeter. The magnification for such drawings was from 700 to 800 times.

MICROFILARIA OF *Filaria malayi.*

In air-dried thick smear preparations *F. malayi* generally appear shrunken or crinkled up, and the large curves of the microfilaria are complicated by irregular waves (Plate XX, figs. 1 to 8). This feature is fairly constant but by no means invariable. On the other hand in living specimens as well as in those preserved wet (for example in Azur II-formalin) the outline is smooth and regular (Plate XIX, fig. *b*), which would indicate that the crinkled appearance and the wavy contour observed in microfilariæ in dry smears is not a natural condition but is due to · shrinkage caused through drying.

The length of the microfilaria varied considerably according to the manner of preservation as shown in Table I :—

TABLE I.

Length of microfilariæ of Filaria malayi.

Material.	Number of observations.	Maximum.	Minimum.	Average.
Azur II-formalin ..	52	299μ	238μ	263·4μ
Dry-smear preparations ..	181	238μ	135μ	185·8μ
Preparations after narcotizing with menthol.	49	285μ	162μ	219·3μ

The marked difference observed in the length of microfilariæ preserved wet and those in air-dried smear preparations would indicate that microfilariæ of *F. malayi* are subject to considerable shrinkage during the drying of the smear. The length of microfilariæ in narcotized preparations was intermediate between those of wet-preserved and of air-dried microfilariæ.

The width of the microfilaria in the region between the nerve ring and the excretory pore varied between 5·0μ and 6·4μ, averaging 5·8μ, in wet material, and between 6·2μ and 8·7μ, averaging 7·5μ, in dried-smear preparations.

The head-end of the microfilaria of *F. malayi* is slightly more pointed than that of *W. bancrofti.* A ring of eight minute teeth-like papillæ is sometimes seen at the head-end in wet-preserved microfilariæ (Plate XXI, fig. 13). The clear

cephalic space is fairly long, being $1\frac{1}{2}$ to 2 times as long as broad (Plate XXI, figs. 7 to 12). Expressed as a percentage of the total length, the cephalic space varied between 2·5 and 4·8 per cent and was on the average 3·7 per cent. The body nuclei behind the cephalic space are massed together and overlapping, and are not easily differentiated from one another, while in *W. bancrofti* the nuclei are generally more evenly spaced.

The nerve ring is on the average at 21·6 per cent of the total length from the head-end. The excretory pore is situated at 29·9 per cent. The excretory cell is placed some distance posteriorly to the excretory pore and is nearly as far away from the excretory pore as the latter is from the nerve ring (Plate XXI, figs. 16 and 17). The excretory cell is large and conspicuous, with a large nucleus and a distinct nucleolus. It has dense cytoplasm with sometimes a few vacuoles. The cytoplasm is continued anteriorly as a protoplasmic strand which connects the excretory cell with the excretory pore (Plate XXII, figs. 1 to 6).

The four G-cells in *F. malayi* (Plate XXIII, figs. 1 to 5) are definitely larger and have bigger nuclei and denser cytoplasm than the corresponding cells in *W. bancrofti*. They are longer than broad and are oval in shape. Cell G-1 which is situated behind the posterior end of the 'Innenkörper' is conspicuous and easily recognized by its large nucleus and well-defined nucleolus. This cell nearly fills the breadth of the microfilaria and is about twice as large as the other G-cells. The cells G-2, G-3, and G-4 occupy positions intermediate between G-1 and the anal pore. Cell G-2 is placed about half-way between G-1 and the anal pore. Generally this cell is slightly nearer to G-1 than to the anal pore. The last of the G-cells, namely G-4, is placed anteriorly to the anal pore and well separated from it.

The anal pore occurs at 80·1 per cent of the total length from the head-end. It is conspicuous and extends from one-half to three-quarters of the breadth of the microfilaria. Its position is clearly indicated by the absence of nuclei of the nuclear column in that region.

The tail in *F. malayi* is characteristic. Beyond the end of the nuclear column the tail enlarges into a bulbous swelling after which it attenuates suddenly and then enlarges gradually into a slightly swollen tip (Plate XXIV, figs. 5 to 10). There are thus two swellings in the tail, one at the tip and another a little above it. In microfilariæ preserved wet the part beyond the anal pore tapers more gradually (Plate XXIV, figs. 5 to 8) than in microfilariæ in dry smears (Plate XXIV, figs. 9 and 10). The more abrupt tapering of the tail-end observed in dry-smear preparations is the result of shrinkage. The shrinkage is, however, not uniform. The terminal portion of the tail beyond the subapical swelling does not shrink appreciably and there is little difference in the absolute length of this segment in wet material and in dry smears. On the other hand, the portion between the anal pore and the subapical bulbous swelling is subject to marked shrinkage like the rest of the microfilariæ. The length of the part between the anal pore and the subapical bulbous swelling in dry-smear specimens is about two-thirds of the length in wet material. A striking feature in *F. malayi* is the presence of nuclei in the terminal part of the tail well beyond the termination of the nuclear column (Plate XXIV, figs. 5 to 10). The position of the tail nuclei is definite; one nucleus occurs in the subapical swelling in the tail and the other in the swelling at

the tip. They readily take stain and are easily recognized in material preserved in Azur II-formalin as well as in smear preparations stained with Giemsa solution or with hæmatoxylin. The occurrence of these nuclei in the tail is a constant feature in *F. malayi.* These nuclei are well separated from the nuclei of the nuclear column and are somewhat different in size and in appearance from the latter. Rodenwaldt (*loc. cit.*) considers that these tail nuclei are not true nuclei but artefacts caused by the deposition of the stain in invaginations and folds of the cuticle. This view is not supported by the observation that the tail nuclei always occur in *F. malayi,* and in the same situation associated with the swellings in the tail. They are as well stained in wet material preserved in Azur II-formalin as in dry smears stained with hæmatoxylin. These nuclei are also present in young larvæ of *F. malayi* within the thoracic muscles of the mosquito vector, *Mansonioides,* prior to the first ecdysis of the worm.

Cuticular striations are not apparent in microfilariæ of *F. malayi* in dry-smear preparations. In material preserved in Azur II-formalin, the striations of the cuticle are sometimes seen faintly, especially in the posterior part of the body. Even where the striation is seen at its best it is not as well marked as that found in *W. bancrofti.*

The length of the sheath in *F. malayi* varied from 248μ to 318μ, averaging 287μ (86 observations). The length of the sheath divided by the length of the microfilaria in wet material (which is here called the 'sheath index') was 1·091* and this figure is smaller than the sheath index of *W. bancrofti.* The tip of the sheath is rounded and sometimes slightly broader than the part just above it. In a few cases I have observed another feeble swelling in the tail-end of the sheath just above the tip corresponding to the swelling in the middle of the tail of the microfilaria.

In microfilariæ of *F. malayi* fixed with absolute alcohol and overstained with Giemsa solution, the sheath took a deep pink colour and its surface frequently presented a furred appearance which was not observed in microfilariæ of *W. bancrofti* similarly stained.

Besides the morphological characters mentioned above, another character which appeared to be fairly constant was that microfilariæ of *F. malayi* failed to develop in the mosquito *Culex fatigans* Wied.† Even when fed on carriers with a large number of microfilariæ in the blood, *Culex fatigans* failed to take the infection in contrast to the ready manner in which it took the infection when fed on blood containing microfilariæ of *W. bancrofti‡.*

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* In the determination of the 'sheath index' the measurements taken into consideration are of microfilariæ preserved *wet* (e.g., in Azur II-formalin). This index would be different if based on measurements in dry preparations.

† The behaviour of the microfilariæ in the mosquito *Culex fatigans* could be used as a confirmatory test but, by itself, it would not give any conclusive evidence. Sometimes, under conditions of low atmospheric temperature and low humidity, it is possible that *W. bancrofti* may fail to infect *Culex atigans.* The possibility of mixed infections should also be borne in mind.

‡ Over 1,300 *Culex fatigans* mosquitoes were fed experimentally on *F. malayi* carriers (Iyengar, 1932, 1938), and except for a solitary specimen which showed young filaria larvæ in the thorax, all the mosquitoes proved to be negative for filarial infection.

MICROFILARIA OF *Wuchereria bancrofti.*

In ordinary thick smears microfilariæ of *W. bancrofti* exhibit smooth curves which are uncomplicated by irregular waves (Plate XX, figs. 9 to 16). The crinkled appearance and corrugations commonly observed in thick-smear preparations of *F. malayi* are generally absent in *W. bancrofti.*

The length of the microfilaria under different methods of preservation is shown in Table II :—

TABLE II.

Length of microfilariæ of W. bancrofti.

Material.	Number of observations.	Maximum.	Minimum.	Average.
Azur II-formalin ..	72	322μ	255μ	290·3μ
Dry-smear preparations ..	69	322μ	256μ	289·1μ
Dried smears after narcotization with menthol.	14	300μ	274μ	287·0μ

The average length in wet preparations was 290·3μ and in dry smears 289·1μ. The absence of any marked difference in the length of microfilariæ preserved wet and of those in dry smears would indicate that microfilariæ of *W. bancrofti* are not subject to any considerable shrinkage consequent on drying of the smear. On the other hand, microfilariæ of *F. malayi* are liable to considerable shrinkage on desiccation as shown by the marked difference observed between the length of microfilariæ preserved wet and of those in air-dried smears*. Thus, while in wet preparations the average length of *F. malayi* is not much less than that of *W. bancrofti*, the difference in the average lengths of the two species in air-dried preparations is very striking†.

The thickness of the microfilaria of *W. bancrofti* in the region between the nerve ring and the excretory pore varied between 5·5μ and 7·7μ and averaged 6·3μ in wet preparations. In air-dried smears it varied between 6·3μ and 8·4μ, and averaged 7·0μ.

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* The presence of well-developed cuticular striations in the case of *W. bancrofti* and the poor development of the striations in *F. malayi* may, perhaps, explain this difference.

† The average lengths in wet preparations of the microfilariæ of *W. bancrofti* and *F. malayi* were 290μ and 263μ respectively, while in air-dried smears the average lengths for the two species were 289μ and 186μ respectively.

The head-end of the microfilaria is broadly rounded. The clear cephalic space is short (Plate XXI, figs. 1 to 6) and forms 1·2 to 2·7 per cent of the total length of the microfilaria, averaging 1·7 per cent. This space is rarely longer than broad. The body nuclei are spaced more evenly than in *F. malayi* and except in shrunken specimens, there is not much of overlapping of the nuclei.

The nerve ring is on the average at 19·0 per cent of the body-length from the head-end. The excretory pore is on the average at 28·5 per cent. The excretory cell has a large nucleus and is placed close behind the excretory pore (Plate XXI, figs. 14 and 15). In some it is adpressed to the wall of the excretory pore, while in others it is placed slightly behind the pore (Plate XXII, figs. 7 to 12). In either case, the position of the cell in relation to the pore is quite different from the arrangement in *F. malayi* in which the excretory cell is well separated from the excretory pore. The distance from the middle of the excretory pore to the middle of the excretory cell is never more than a fourth of the distance between the excretory pore and the nerve ring. On the anterior side of the excretory cell a short and thick cytoplasmic process connects it with the excretory pore. Sometimes one or two vacuoles may occur in the cytoplasm. On the posterior side the cytoplasm is drawn out as a long, thin, and sinuous strand measuring about 15μ to 20μ long.

The G-cells in *W. bancrofti* are smaller in size than those of *F. malayi* and the cytoplasm is less dense. The cells are not longer than broad and are often angular in outline (Plate XXIII, figs. 6 to 10). Frequently one side of the cell is flat. Cell G-1, situated behind the end of the 'Innenkörper', is inconspicuous and is not much larger than the other G-cells. The cells G-2, G-3, and G-4 are placed close to one another and near the anal pore. The distance between cell G-2 and the anal pore is about a fourth (or a third) of the distance between G-1 and the anal pore. Cell G-4 is placed very close to the anal pore. Its position in relation to the anal pore is somewhat variable. Generally it is placed anteriorly to the anal pore but less frequently it occurs at the same level as the anal pore.

The anal pore occurs on the average at 81·1 per cent of the total length from the head-end. It is small and inconspicuous, and does not cause a break in the distribution of the nuclei of the nuclear column.

The tail tapers gradually and ends in a blunt tip (Plate XXIV, figs. 1 to 4). There are no swellings at the middle of the tail or at its tip. There are no nuclei in the tail beyond the termination of the nuclear column.

In microfilariæ of *W. bancrofti* the cuticular striations are seen very clearly in wet preparations (Azur II-formalin) and in thin smears stained with Giemsa solution. The striations can be observed even in ordinary thick-smear preparations though not as clearly as in wet preparations.

The sheath of the microfilaria of *W. bancrofti* is definitely longer than that of *F. malayi*. It measures from 334μ to 402μ, averaging 358·6μ (21 observations). The sheath index (length of sheath divided by length of microfilaria in *wet* material) is 1·235.

Culex fatigans mosquitoes when fed on blood containing microfilariæ of *W. bancrofti* become infected very readily in contrast with the negative results obtained with *F. malayi*.

The important points in the differentiation of the microfilariæ of the two species are shown in Table III :—

TABLE III.

Comparison of microfilariæ of Filaria malayi *and* Wuchereria bancrofti.

Character.	*Filaria malayi.*	*Wuchereria bancrofti.*
Length in wet material ..	238μ to 299μ, average 263μ.	255μ to 322μ, average 290μ.
Length in smear preparations.	135μ to 238μ, average 186μ.	256μ to 322μ, average 289μ.
Breadth, wet ..	5·8μ.	6·3μ.
,, dry ..	7·5μ.	7·0μ.
Appearance in thick smears.	Shrunken; cuticle often corrugated; irregular curves.	Not shrunken; smooth curves.
Cuticular striations ..	Not noticeable except in wet preparations in which they are sometimes seen faintly.	Well marked.
Cephalic space ..	1½ to 2 times as long as broad.	Generally less than breadth.
Excretory cell ..	Well separated from excretory pore and nearly as far away from it as the latter is from nerve ring.	Adjoins excretory pore.
G-cells	Large, oval, with dense cytoplasm.	Smaller, rounded, stellate or angular; cytoplasm not dense.
Cell G-1	Large, nearly fills breadth of microfilaria; twice as large as other G-cells.	Small, about same size as other G-cells.
Cell G-2	Situated about half-way between anal pore and G-1.	About ¼th way between anal pore and G-1.
Cell G-4	Well separated from anal pore and placed anterior to it.	Very close to anal pore and placed slightly anterior to it or at the same level.
Anal pore	Conspicuous.	Not conspicuous.
Tail	Tail with two swellings, one at tip and another just above it.	No swelling in tail. Tail tapers gradually to a blunt tip.
Terminal tail nuclei ..	Two small nuclei present in tail beyond end of nuclear column.	No nuclei beyond termination of nuclear column.
Length of sheath ..	248μ to 318μ, average 287μ.	334μ to 402μ, average 359μ.
Development in *Culex fatigans*.	No development.	Normal development.
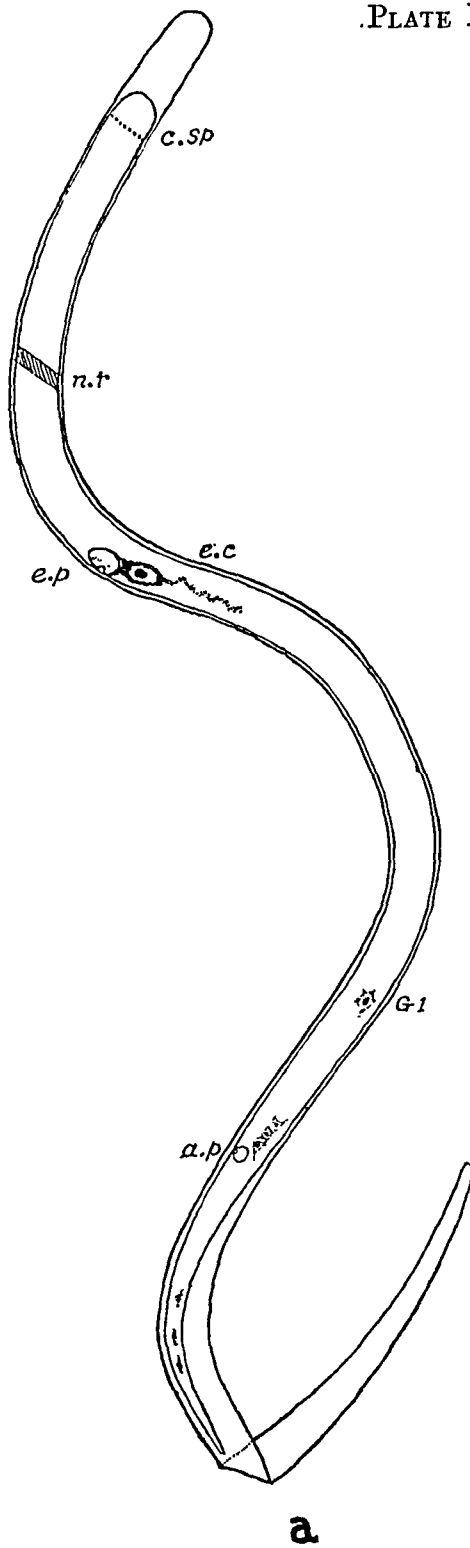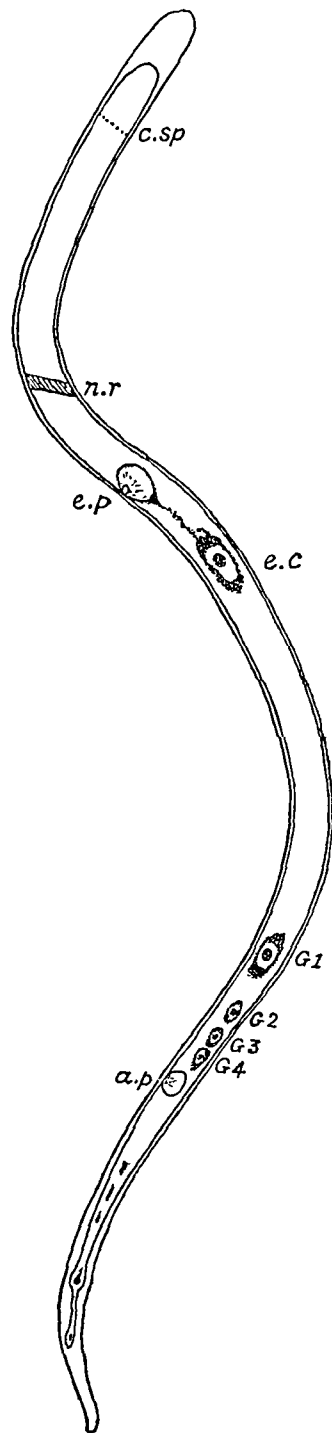
Fig. *a*. Microfilaria of *Wuchereria bancrofti*.   Fig. *b*. Microfilaria of *Filaria malayi*.

Reconstructed from a series of camera-lucida drawings made of material preserved in Azur II-formalin.

c.sp.	Cephalic space.	*G*-1		
n.r.	Nerve ring.	*G*-2		
e.p.	Excretory pore.	*G*-3	}	The four G-cells.
e.c.	Excretory cell.	*G*-4		
a.p.	Anal pore.			

PLATE XX.
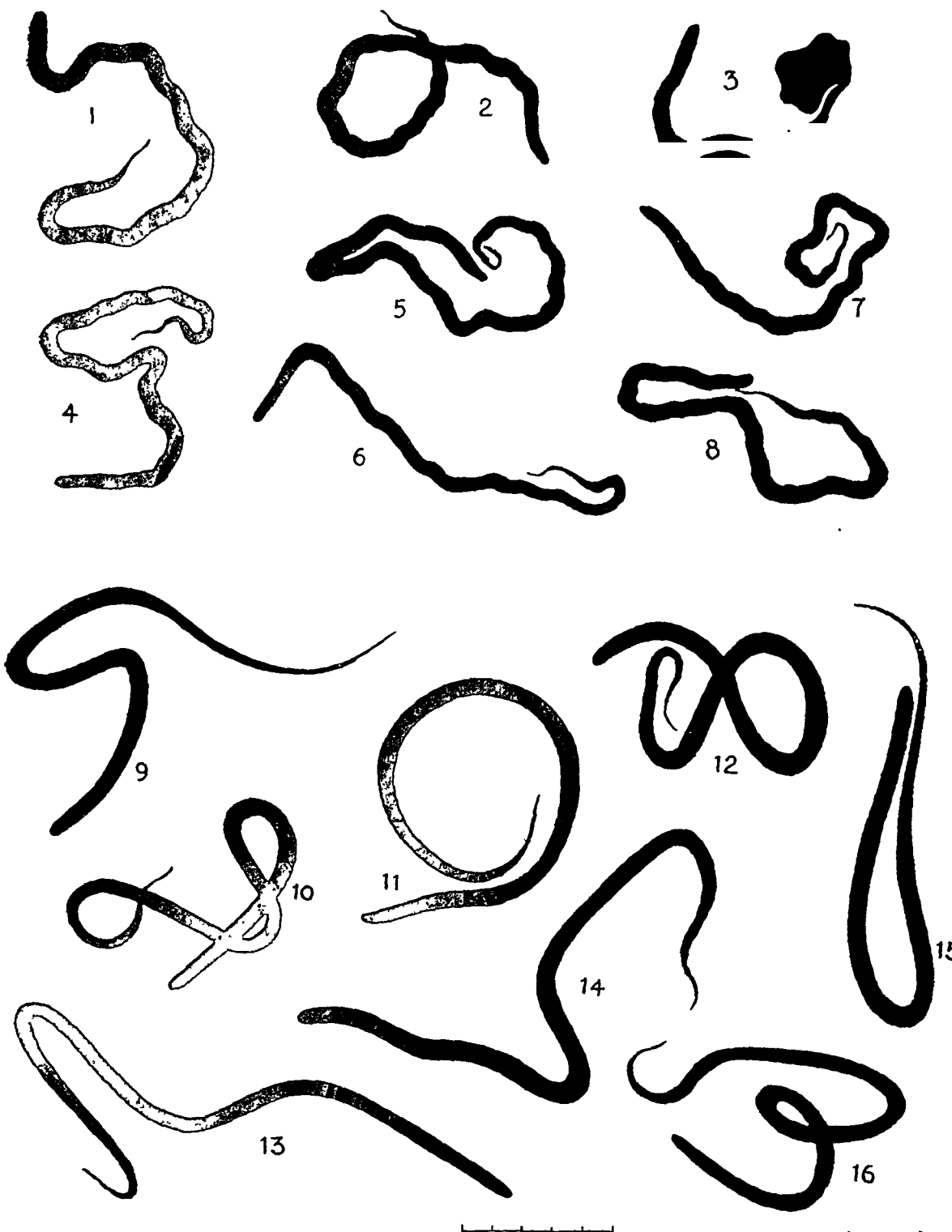
General lie of microfilariæ in air-dried thick smears.
Figs. 1 to 8. *Filaria malayi.*
Figs. 9 to 16. *Wuchereria bancrofti.*
Camera-lucida drawings. Each division of the scale represents 10μ.

PLATE XXI.
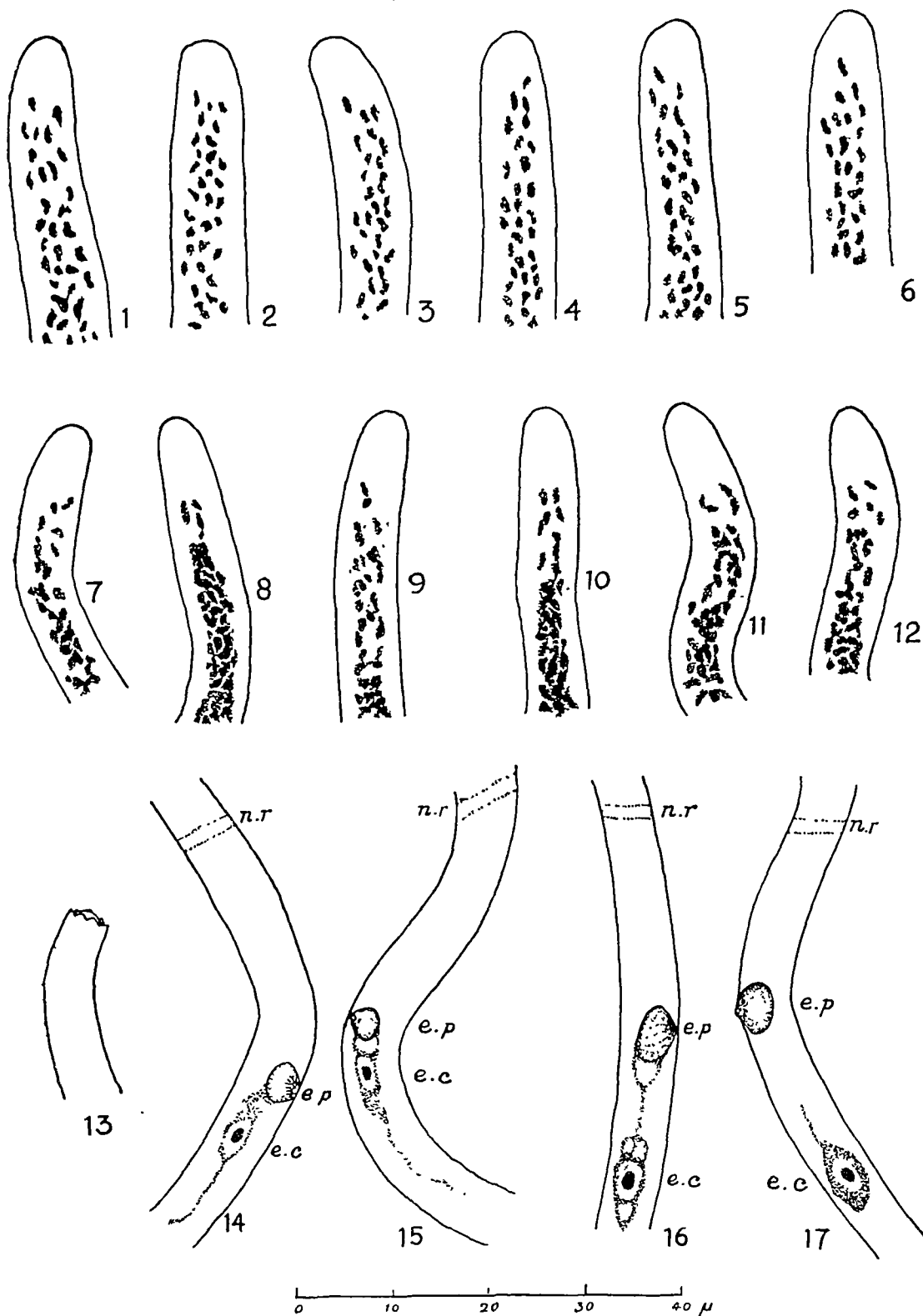
Figs. 1 to 6. Head-end of microfilariæ of *W. bancrofti* showing cephalic space and distribution of nuclei. (Air-dried smears stained with hæmatoxylin.)

Figs. 7 to 12. Head-end of microfilariæ of *F. malayi*. (Air-dried smears stained with hæmatoxylin.)

Fig. 13. Head-end of *F. malayi* in a wet preparation showing ring of papillæ.

Figs. 14 and 15. Nerve ring, excretory cell and excretory pore in *W. bancrofti*. (Azur II-formalin material.)

Figs. 16 and 17. Nerve ring, excretory pore and excretory cells in *F. malayi*. (Azur II-formalin material. Camera-lucida drawings.)

PLATE XXII.
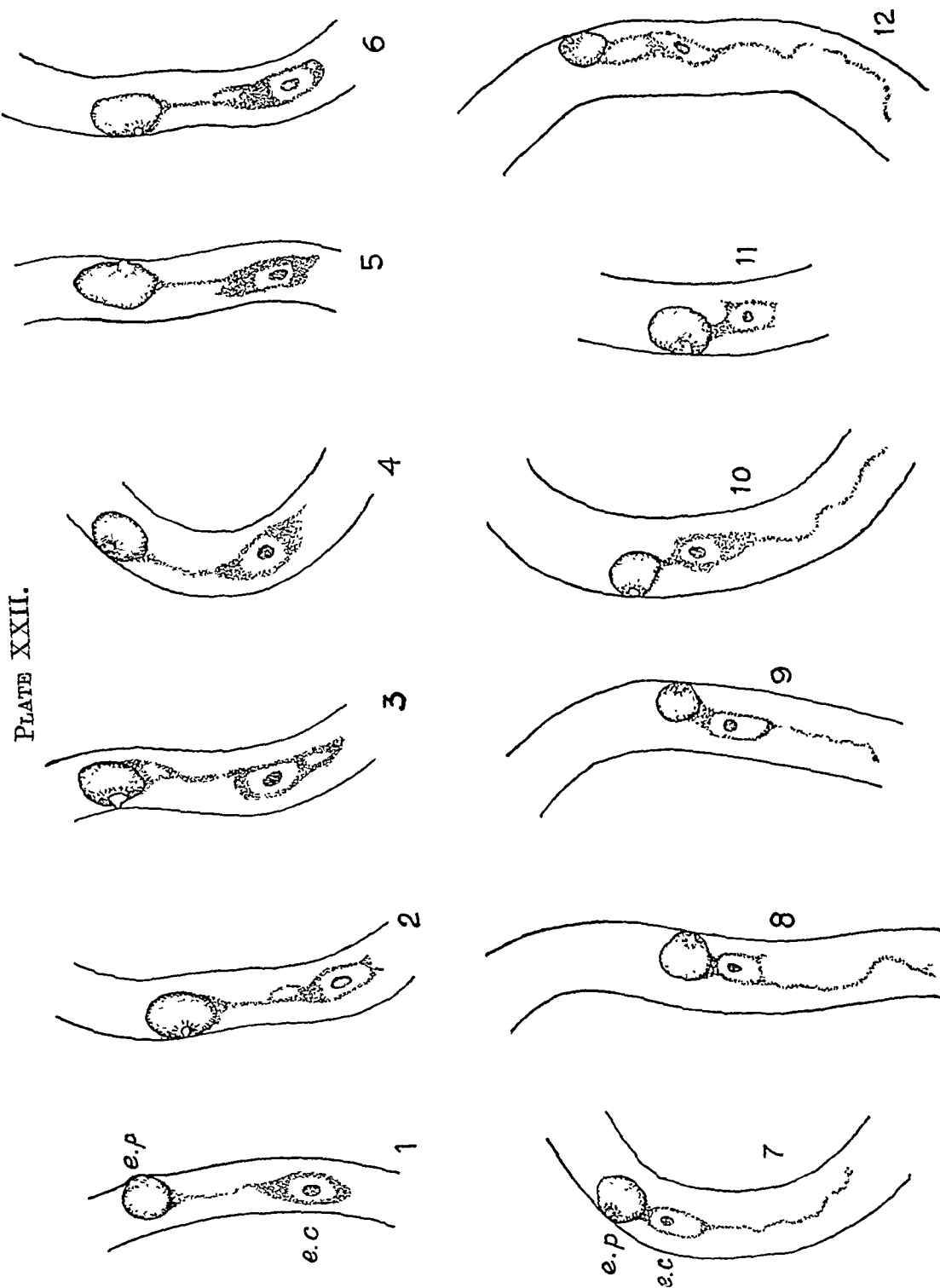
Figs. 1 to 6. Excretory pore and excretory cell in *F. malayi*.
Figs. 7 to 12. Excretory pore and excretory cell in *W. bancrofti*.
(Camera-lucida drawings. Azur II-formalin material.)

PLATE XXIII.
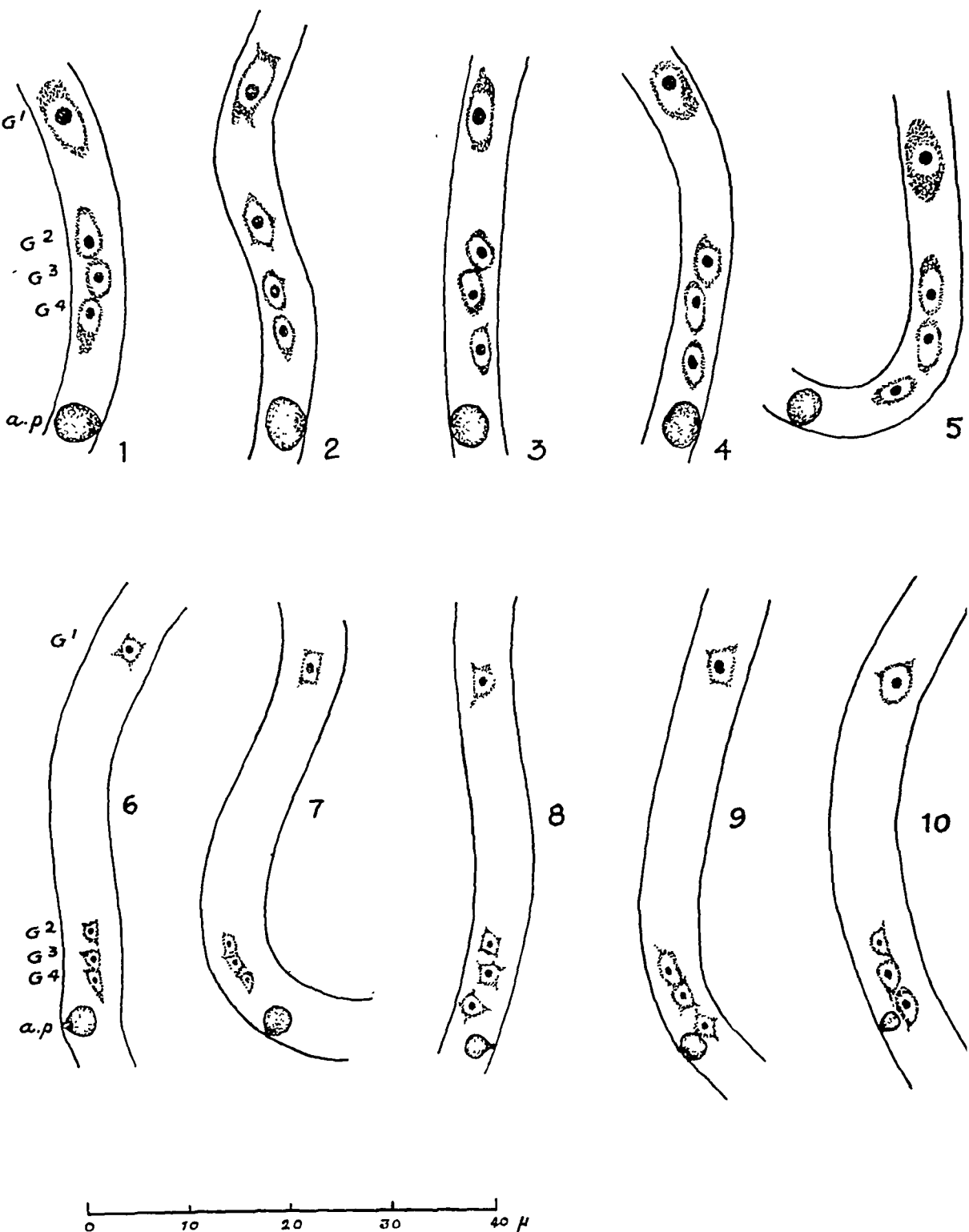
Figs. 1 to 5. The G-cells and anal pore in *F. malayi*.
Figs. 6 to 10. The G-cells and anal pore in *W. bancrofti*.
(Camera-lucida drawings. Azur II-formalin material.)

PLATE XXIV.
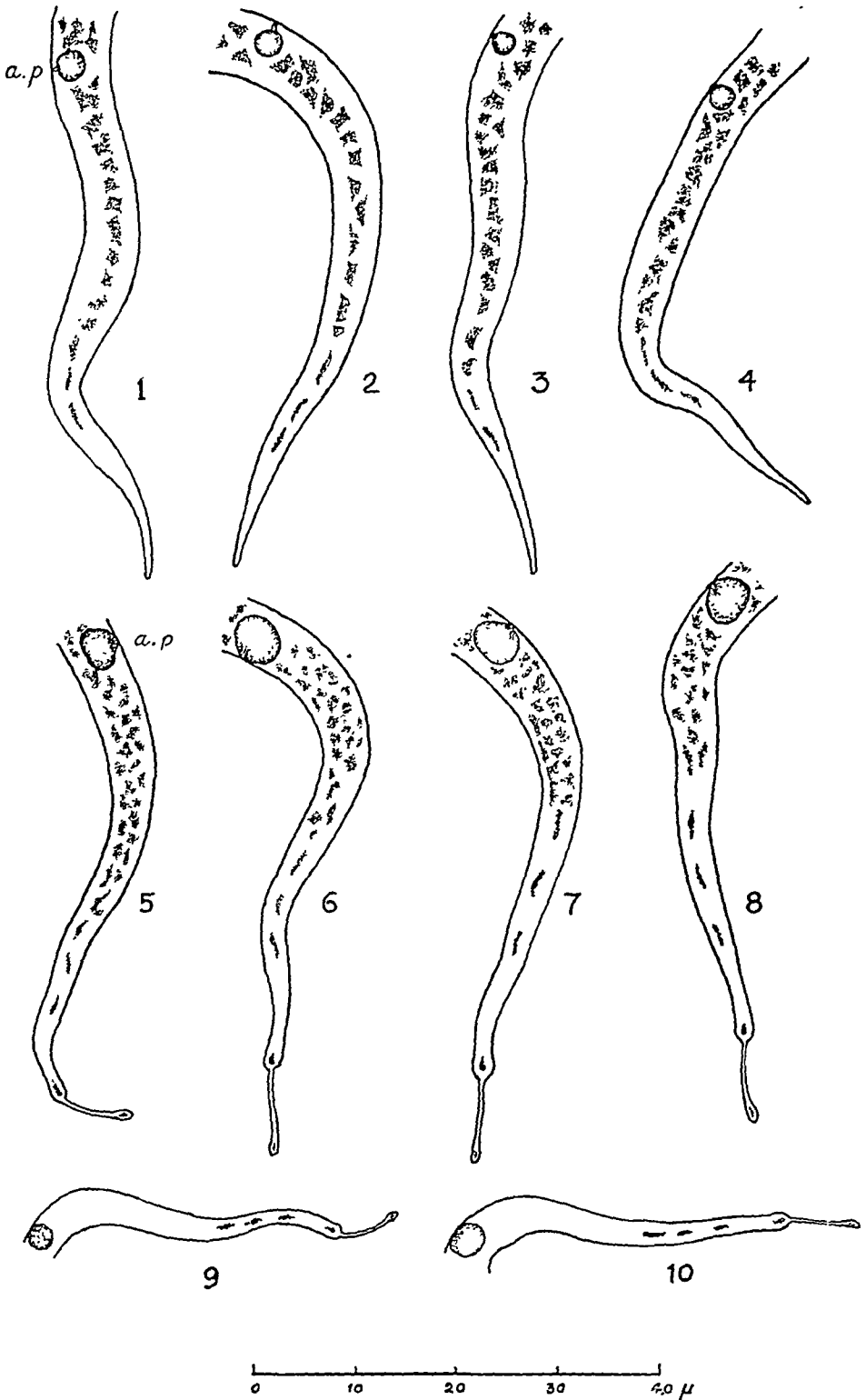
Figs. 1 to 4. Tail-end of microfilariæ of *W. bancrofti*. (Azur II-formalin material.)
Figs. 5 to 8. Tail-end of microfilariæ of *F. malayi*. (Azur II-formalin material.)
Figs. 9 and 10. Tail-end of *F. malayi*, from thick smears stained with hæmatoxylin.
(Camera-lucida drawings.)

SYNONYMY OF *Filaria malayi* BRUG.

In an editorial note in the *Ind. Med. Gaz.*, 1937, Vol. **72**, p. 734, an opinion was expressed that it was inadmissible to place this species under the genus *Filaria* as it had been described only from the larval form, and it was suggested that it should be termed *Microfilaria malayi*. The term *Microfilaria* is not a systematic unit and is therefore not a ‘ genus ’ (*vide* Baylis and Daubney, 1926). Like the term *Agamofilaria*, it is only a collective group meant for the sake of convenience to accommodate species of *Filariidæ* described from larval forms in cases where the authors are doubtful of the generic position. Since, however, the author of the species *Filaria malayi* had placed it under the genus *Filaria*, there is no justification now for changing the name to *Microfilaria malayi*, merely because it was described from the larval form. There appears to be no convincing objection to continue to call it by the name under which it was originally described until such time as the adult stages are discovered when its generic position would be revised if found necessary.

Under the name *Filaria malayi* Brug, I have to sink *Microfilaria actoni* Rao, 1931, as a synonym. Through the courtesy of Dr. S. Sundar Rao, I have examined the single preparation from which he described *Microfilaria actoni*. In the original preparation the sheaths were not clearly seen and consequently the microfilaria was described as sheathless. The preparation has since been stained with hæmatoxylin and shows the sheaths clearly. Morphologically I can find no difference between the microfilariæ in Dr. Rao’s preparation and those of *F. malayi*.

The ‘ atypical forms of *W. bancrofti* ’ of Korke (1929) are also synonymous with *F. malayi* as judged from Korke’s description and figures.

ACKNOWLEDGMENTS.

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FLOCCULATION TEST WITH *CLOSTRIDIUM SEPTIQUE* TOXIN AND ANTITOXIN.

BY

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IT was first observed by Ramon (1922*a*) that, in a series of mixtures of diphtheria toxin and specific antitoxin, the neutral or balanced mixture flocculates more quickly than the others and the reaction is delayed or inhibited when either of the reacting ingredients is in relative excess. Since his classical observation much useful work has been performed by numerous observers to study the manifold aspects and implications of this precipitation reaction. Many different antigens and their specific antibodies were studied and doubts were raised, whether the ' constant antigen-optimum ratio' of Ramon really corresponds to the neutral point of the antigen-antibody mixtures. Dean and Webb (1926) were the first to apply the reverse technique of ' constant antibody optimum ratio' titration, with horse serum as antigen, and the serum obtained by immunizing rabbits against horse serum, as antibody. Their technique of ' constant antibody' titration was followed by Smith (1932) with specific soluble substance from type I pneumococcus as antigen, by Duncan (1932) with polysaccharide extracted from some fungi and by Taylor (1933) with crystalline egg-albumin and the respective antisera. Duncan (*loc. cit.*) and Taylor (*loc. cit.*) demonstrated by testing the supernatant fluids from their series of antigen-antibody mixtures that the Dean and Webb's ratio actually corresponded with the balanced mixture. They also observed that the ' constant antigen O. R. ' and the ' constant antibody O. R.' were at marked variance. These observations could not be easily reconciled with the Ramon's method of antitoxin titration, though the close agreement of the results of flocculation tests by the ' constant antigen ' technique and the *in vivo* experiments, justified the presumption that, the point of Ramon's flocculation of toxin and antitoxin is also the point of chemical equivalence between these reagents. Since Miles (1933) has shown that the ' constant antigen O. R.' and the ' constant antibody O. R.' with mixtures of toxin and antitoxin differ very little, especially with stronger toxins, the apparent discrepancy has lost much of its practical importance.

In the present paper the author intends to put forth the results of his study of the flocculation reaction with *Cl. septique* toxin and antitoxin by the Ramon's

'constant antigen' technique. Several samples of antiserum have been titrated by the *in vitro* and the *in vivo* methods. The results have been shown in Table III.

<center>EXPERIMENTAL.</center>

The flocculation tests were performed with liquid toxoid, while for the animal titrations ammonium-sulphate-precipitated solid toxin was used, as the toxic filtrate rapidly loses its toxicity on keeping.

Titration of unknown antiserum by the antidermonecrotic test on guinea-pig.—The technique for the preparation of dry toxin, the determination of $\frac{L\,R}{10}$ dose and assay of unknown antitoxin has been described in a previous paper by Basu, Roy and Ghosh (1938). The method of titration was fundamentally the same as that recommended by the Health Organization of the League of Nations (1935), though varying in detail. The $\frac{L\,R}{10}$ dose of the dry toxin lot 17 on guinea-pig was found to be 0·85 mg.

Different samples of *Cl. septique* antitoxin were titrated against this $\frac{L\,R}{10}$ dose. The results have been put in column 8 of Table III.

<center>*Ramon's flocculation test with* Cl. septique *toxoid.*</center>

Determination of L_f dose of toxoid.—In each of a series of test-tubes of uniform shape and bore (6 mm. × 120 mm.) 1 c.c. of toxoid was taken with a tuberculin syringe. Standard *Cl. septique* antitoxin, suitably diluted, was then added in falling quantities and the volume in each tube was made up to the total volume in the tube that received the maximum quantity of antitoxin with normal saline. The contents in each tube were well mixed and the rack put in the water-bath at 50°C. A preliminary test with a wide range of the series of antitoxin units added with a comparatively wide difference between the adjacent tubes was performed. A shorter range with less difference in antitoxin units in adjacent tubes was taken in the final testing, on the basis of preliminary test. Each test was repeated more than once for confirmation. The number of antitoxin units present in the first flocculating tube was taken as the number of L_f doses present in 1 c.c. of the toxoid. The actual test has been shown in Table I.

Titration of unknown antitoxin by the flocculation test.—The technique is essentially the same as that for determination of L_f dose of toxoid. To a constant volume of toxoid (2 c.c.) in a series of test-tubes falling quantities of a suitable saline dilution (1 in 5, when the expected titre was low and 1 in 10, when higher titre was expected) of the unknown antiserum was added and the volumes made equal with normal saline. The titre of the sample of antiserum was obtained from the actual volume of serum and the volume of toxoid present in the first flocculating tube, since the number of L_f doses present in one c.c. of the toxoid was known from the previous experiment (*vide supra*). Here also, as in case of determination of L_f dose of toxoid, a preliminary test was followed by repeated more accurate titrations. Table II shows an example of titration of an unknown antitoxin by the flocculation test.

TABLE I.

Determination of L_f dose of toxoid lot 40.

	Tube number.	1	2	3	4	5	6	7	8	9	L_f doses per c.c. of toxoid.
Preliminary test. {	Toxoid lot 40	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	..
	Standard antitoxin (20 units) per c.c.	0·1 c.c.	0·2 c.c.	0·3 c.c.	*0·4 c.c.*	0·5 c.c.	0·6 c.c.	0·7 c.c.	0·8 c.c.	0·9 c.c.	..
	Total volume ..	1·9 c.c.	1·9 c.c.	1·9 c.c.	1·9 c.c.	1·9 c.c.	1·9 c.c.	1·9 c.c.	1·9 c.c.	1·9 c.c.	Between 6 and 10
Final test {	Toxoid lot 40	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	..
	Standard antitoxin (10 units) per c.c.	0·6 c.c.	0·65 c.c.	0·7 c.c.	0·75 c.c.	0·8 c.c.	*0·85 c.c.*	0·9 c.c.	0·95 c.c.	1 c.c.	..
	Total volume ..	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	8·5

Italicized figures indicated the first flocculating mixtures.

TABLE II.

Titration of unknown antiserum A₁ by flocculation test.

		Tube number.	1	2	3	4	5	6	7	8	Titre.
Preliminary test	{	Toxoid lot 40	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	
		Antiserum A₁-diluted (1 in 10).	0·3 c.c.	0·4 c.c.	0·5 c.c.	0·6 c.c.	0·7 c.c.	0·8 c.c.	0·9 c.c.	1 c.c.	Between 212 and 283 units per c.c.
		Total volume ..	3 c.c.	3 c.c.	3 c.c.	3 c.c.	3 c.c.	3 c.c.	3 c.c.	3 c.c.	.. ..
Final test	{	Toxoid lot 40	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.				
		Antiserum A₁-diluted (1 in 10).	0·6 c.c.	0·65 c.c.	0·7 c.c.	0·75 c.c.	0·8 c.c.	.. ..	.. ..	.. ..	243 units per c.c.
		Total volume ..	2·8 c.c.	2·8 c.c.	2·8 c.c.	2·8 c.c.	2·8 c.c.	..	..	..	

Italicized figures indicated the first flocculating mixtures.

Results of assay of 12 samples of antitoxin by the antidermonecrotic test on guinea-pig and by the Ramon's flocculation test against three different lots of toxoid have been arranged side by side under Table III for comparison :—

TABLE III.

Results of titration of different samples of antitoxin by the in vitro *and* in vivo *methods.*

Antitoxin number.	FLOCCULATION TEST WITH TOXOID LOT 37 (6 Lf DOSES PER c.c.).		FLOCCULATION TEST WITH TOXOID LOT 40 (8·5 Lf DOSES PER c.c.).		FLOCCULATION TEST WITH TOXOID LOT 41 (8 Lf DOSES PER c.c.).		ANTIDERMONECROTIC TEST WITH DRY TOXIN LOT 17 $\left(\frac{LR}{T0} \text{ DOSE}=0.85 \text{ mg.}\right)$.	
	Observed titre.	Limits of titre tested for.	Observed titre.	Limits of titre tested for.	Observed titre.	Limits of titre tested for.	Observed titre.	Limits of titre tested for.
1	2	3	4	5	6	7	8	9
A_1 ..	240	218,266	243	227,262	246	229,267	240	220,260
A_2	171	164,184	179	162,200	178	168,200	180	160,200
B_1	200	185,218	200	189,212	200	188,213	200	180,220
B_2	80	75,86	81	73,89	80	73,89	80	70,90
C_1	120	109,133	121	106,142	123	114,133	120	110,130
C_2	266	240,300	262	243,283	267	246,291	260	240,280
C_3	240	218,266	243	227,262	246	229,267	250	225,275
C_4	67	60,75	71	61,85	67	62,73	70	60,80
D_1	150	144,160	155	142,170	145	133,160	160	140,180
D_2	133	120,150	142	131,155	133	123,145	140	120,160
$P.P_1$	218	200,240	227	200,262	229	200,267	220	200,240
Concentration$_1$ (in normal saline).	*Nil*	..	*Nil*	..	..	..	260	240,280
Concentration$_1$ (in sodium citrate solution of molar concentration 0·17).	266	240,300	262	243,283	..	..	..	..

The figures indicate the titre in units per c.c. The figures in columns 2, 4, and 6 were obtained from the first flocculating tubes and those in column 8 from the mixtures that just failed to produce necrosis in the guinea-pig. Mixtures in the respective series that immediately preceded and followed the above-mentioned mixtures, gave the figures in columns 3, 5, 7, and 9. When on calculation the titre came to a fraction of one, the nearest whole number was accepted.

The sample P.P$_1$ is a mixture of sera obtained from four different horses. The sample concentration$_1$ is the so-called pseudoglobulin fraction of a mixed serum from two different horses, obtained by fractional precipitation with ammonium sulphate. All other samples were unconcentrated sera obtained from single horses at different stages of immunization. It is interesting to note (*vide* Table III) that, the sample concentration$_1$ did not give any flocculation with toxoids lots 37 and 40, when normal saline was used as menstruum. This is in agreement with Ramon's (1922*b*) findings with diphtheria toxin and the concentrated antitoxin. Eagle (1935) has shown that, this failure of flocculation does not mean that combination of the reagents does not take place. Ghosh and Ray (1937) have obtained specific flocculation reaction with tetanus and diphtheria toxins and the ammonium sulphate precipitated specific pseudoglobulins by changing the electrolyte in the menstruum. It will be noted from Table III that the antitoxin concentration$_1$ gave flocculation reaction with toxoids lots 37 and 40 near the point of neutralization determined by the *in vivo* experiment, when sodium-citrate solution of molar concentration 0·17 was used in place of normal saline as menstruum. Unfortunately, only a small quantity of a single sample of concentrated antitoxin was available and the effects of electrolytes could not be studied further. Very recently, Weil and Parsons (1939) have obtained specific flocculation using *Cl. welchii* toxin concentrated by ultrafiltration as antigen and unrefined antiserum as well as antiserum refined by Parfentjev's method of partial peptic digestion as antibody. Unconcentrated *Cl. welchii* toxin has failed to produce any flocculation.

SUMMARY.

1. Eleven samples of unconcentrated *Clostridium septique* antitoxin were titrated by the guinea-pig intradermal method and by the Ramon's flocculation test using three different lots of toxoid. The results were found to tally with reasonable accuracy.

2. The only sample of concentrated antitoxin tested, obtained by fractional precipitation with ammonium sulphate from a mixed lot of antiserum from two different horses, failed to give any flocculation when normal saline was used as menstruum, while with sodium-citrate solution of molar concentration 0·17 flocculation was obtained in the region of the *in vivo* titre.

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ABSENCE OF SPECIFIC ANTITOXIN IN PERSONS EXPOSED TO RISK OF TETANUS INFECTION.

BY

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It is known that normal human beings may contain certain antitoxins in their blood naturally, and that they are immune to certain infections by virtue of their possessing these specific antitoxins. Many individuals in a population contain diphtheria antitoxin in their blood (Zingher, 1923). Similarly, scarlatinal antitoxin is also naturally present in many individuals (Zingher, 1924). Recently, Ramon *et al.* (1936) have shown that staphylococcus antitoxin is present in human beings naturally. In contrast, the finding of Ramon (1936) that tetanus antitoxin of natural origin is not found in men, appears to be striking.

A study of the frequency of distribution of persons containing in their blood natural diphtheria and scarlatinal antitoxins in various age groups, or groups with varying changes or durations of exposure to infection, suggests with certain exceptions a correlation between exposure to infection and the specific immunity acquired naturally (*see* Topley and Wilson, 1936). Hence it was considered interesting to find out whether any tetanus antitoxin is present in the serum of persons very highly exposed to the risks of infection.

EXPERIMENTAL.

Selection of subjects.—Of the total of twenty-five persons selected for the experiment, twenty were syces constantly attending for more than three years to horses used for the production of therapeutic sera. They walk bare-footed often with cuts, pricks, and abrasions throughout the yard used by the horses which is notorious for the presence of toxigenic strains of *Cl. tetani*. Many of them were violently injured accidentally in the stable and received prophylactic antitoxin injections, but none within one month before the date of experiment. No one of them suffered from tetanus.

The other five were laboratory workers, who handle tetanus toxin or formol toxoid and cultures of *Cl. tetani* almost daily. Three of them accidentally got cuts or injuries of the fingers while at work during the last year and had antitoxin

injections.   One of them got a deep wound contaminated with pure culture of *Cl. tetani* in cooked meat medium and received four injections of 3,000 I.U. of antitoxin weekly—the last one about a month and a half before the date of the experiment.

Method of experiment.—Two c.c. of blood were drawn from each of the subjects on the same day.   Serum was separated and cleared by centrifuging.

The toxin broth selected for the experiment was filtered nine days before the date of the experiment and stored in a flask with rubber-stoppered mouth in the ice-chest.   The minimal lethal dose of the toxin-broth for guinea-pigs was about 1/15,000 c.c., and its L_+ dose against 0·1 I.U. of antitoxin was about 1/42 c.c. in guinea-pigs.

0·6 c.c. of each serum was mixed with 0·6 c.c. of 1/4,000 dilution of the toxin-broth.   The mixtures were incubated for half an hour at 37°C. and then one c.c. of mixture was injected subcutaneously in a guinea-pig for each sample.   0·5 c.c. of 1/4,000 dilution, one c.c. of 1/15,000 dilution, and one c.c. of 1/20,000 dilution of the toxin-broth were injected in two guinea-pigs for each dilution for control test of toxicity of the toxin-broth.

Result of the experiment.—All the guinea-pigs injected with serum-toxin mixtures developed contracture in 24 hours.   They died between 72 and 84 hours after injection.   One of the control guinea-pigs injected with 1/4,000 dilution of the toxin-broth died after 48 hours and the other between 48 and 60 hours after injection.   Of the two guinea-pigs injected with 1/15,000 dilution of the toxin-broth, one of them died between 72 and 84 hours and the other 94 hours after injection. Both of the guinea-pigs injected with 1/20,000 dilution of the toxin-broth were living till five days of observations, though both of them developed contracture.

DISCUSSION AND CONCLUSION.

0·5 c.c. of 1/4,000 dilution of the toxin-broth contained approximately two minimal lethal doses for guinea-pigs.   0·5 c.c. of serum from different persons could not neutralize it, whereas 0·1 I.U. of antitoxin neutralized about 350 minimal lethal doses of this particular toxin-broth.   So it may be concluded that there is not even a trace of tetanus antitoxin in the serum of any of the persons examined.   Serum appears to have somewhat prolonged the time of death of guinea-pigs in comparison with that of controls injected with the same amount and dilution of the toxin-broth. But this action seems to be non-specific from the uniformity of the degree of delay effected by sera from different persons, and does not possibly signify any presence of tetanus antitoxin in the sera.

Perhaps a reasonable explanation for the absence of tetanus antitoxin in the circulation of the selected subjects is that there is possibly no sub-clinical form of tetanus infection.   The subjects might have been frequently infected with *Cl. tetani*.   But the organisms could not possibly multiply either due to prophylactic antitoxin injections or due to want of suitable conditions required for the germination of the spores.   Hence no toxin could have been formed by them, and so consequently no antitoxin developed in the subjects.   Had there been any multiplication of the organisms in the infected tissues with the liberation of toxin, possibly clinical tetanus would have resulted.

Alternatively, it may be assumed that in some cases the organisms did possibly multiply to a limited extent and liberated small amounts of toxin insufficient to cause clinical tetanus. The infection-resisting mechanism did not, however, respond to it with the formation of antitoxin. The defect appears to depend on insufficient quantity of the toxin formed to serve as a suitable stimulus, rather than on the quality of the infection-resisting mechanism as it is known that injection of tetanus toxoid in man results in the appearance of considerable amounts of tetanus antitoxin in the circulation (*see* Ramon, 1937). On this assumption it would appear that an amount of toxin formed by the infecting organism which is insufficient to cause the disease is also inefficient as a stimulus for the formation of antitoxin by the infection-resisting mechanism.

SUMMARY.

Examination of twenty-five persons very highly exposed to the risk of infection with *Cl. tetani* for a long time showed complete absence of tetanus antitoxin in their serum.

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THE COMPLEMENT-FIXATION TEST IN TUBERCULOUS DISEASE OF THE SKIN.

BY

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SINCE Widal first experimented with the Bordet-Gengou reaction in tuberculosis, using a suspension of living tubercle bacilli as antigen, the test has been tried by numerous workers with conflicting results. In 1914 Besredka detected specific antibodies by a complement-fixation test with the antigen prepared by him, and since then a good deal of work has been performed on the test in tuberculosis by various workers with the same antigen. Hruska, Pfenninger, Brocq-Rousseu and Urbain applied the test for the detection of specific antibodies in tuberculous cattle and the first two authors concluded that the fixation test was destined to render important service in the fight against bovine tuberculosis.

Mozer and Fried, in 1921, found specific antibodies in both surgical (bone and joint) and pulmonary tuberculosis by complement-fixation. In 1924 Fried concluded that complement-fixation in pulmonary tuberculosis with Besredka's bacillary emulsion antigen is specific and Wadsworth, Maltaner and Stevens (1930) corroborated the above finding in a series of 1,002 cases.

More recently, Zeyland and Piasecka-Zeyland did the complement-fixation test in pulmonary tuberculosis and reached the conclusion that it is of value for diagnosis, for it gave positive results in 80 per cent of cases.

Very little work has so far been done with regard to the complement-fixation test in tuberculous diseases of the skin compared with the amount of work in pulmonary tuberculosis, and none at all has been done in India previously.

Von Schönfeld (1934) tried complement-fixation in both pulmonary and skin tuberculosis and concluded that the test had a definite significance in the former,

but in the latter. particularly in lupus vulgaris, the positive reaction had little value in diagnosis as he got somewhat similar results in other skin diseases such as lupus erythematosus, seborrhœic eczema, neurodermatitis, psoriasis vulgaris, acne vulgaris, and syphilis.

Schreus (1936), in studying the complement-fixation in lupus vulgaris, said that there was a low percentage of positive reactions compared with active lung tuberculosis. He also said that the presence of a large number of tubercle bacilli was necessary for a positive tuberculous reaction.

Salmenkallio (1937) studied the complement-fixation test of 1,324 samples of blood of which eighteen were from cases of lupus vulgaris. The percentage of positive reactions in these cases was very low.

More recently, Brandt and Kien (1938) remarked from their serological investigation of tuberculosis that the types of skin tuberculosis showed a varying percentage of positive results and the serological test might be valuable for diagnostic purposes in some cases.

PRESENT INVESTIGATION.

This was carried out on similar lines to those described by Lowe and Greval (1939) and Greval *et al.* (1939).

Technique.

Antigen.—The antigen prepared from tubercle bacilli by the Witebsky, Klingenstein and Kuhn (W. K. K.) method, was used in the test as this is easily available in the market. A measured quantity of the antigen was poured into a porcelain basin, the benzol was evaporated over a water-bath at 60°C., and the basin was kept at 37°C. in an incubator for 10 to 15 minutes for thorough drying. The dried antigen was then carefully emulsified with the addition of double the quantity of normal saline by means of a glass pestle, the saline being added drop by drop to get a perfect suspension. From this suspension dilutions of 1 in 10 and 1 in 20 were prepared and tested for anti-complementary action each time before use.

Hœmolytic system.—This was kindly supplied by the Imperial Serological Laboratory. There it is prepared in accordance with the requirements of method No. IV of the Medical Research Committee's (1918) report on the Wassermann Test with the exception that the red corpuscle suspension was standardized by a method described by Greval *et al.* (1930).

Complement.—The m. h. d. of the complement determined for the Wassermann reaction in the above laboratory was used in our tests.

Serum.—Blood was collected under aseptic precautions from patients suffering from lupus vulgaris, tuberculosis verrucosa cutis, scrofulodermia, and tuberculide, which were diagnosed both clinically and by histopathological study of individual cases, and from patients suffering from other non-tuberculous skin diseases as controls (*see* Table). The serum was separated after being kept with the clot in the ice-chest for about 24 hours. It was inactivated for half an hour at 56°C. and tested for anti-complementary action.

The time and quantities used in the test.

0·25 c.c. of antigen diluted either 1 in 10 or 1 in 20, according to the non-anti-complementary action in different stocks of the W. K. K. antigen, 0·25 c.c. of inactivated serum diluted 1 in 5 in normal saline and 0·25 c.c. of the complement dilution containing 2 m. h. d. were put in one test-tube and kept at room temperature for half an hour, then in an incubator at 37°C. for another half hour. After this period 0·25 c.c. of the hæmolytic system was added and the whole was kept again in the incubator for half an hour and the reading taken. The results are shown in the Table :—

TABLE.

Disease.	Number of cases.	Positive.	Slightly positive.	Doubtful.	Negative.	Percentage positive.
Lupus vulgaris.. ..	37	2	3	2	30	5·4
Tuberculosis verrucosa cutis	31	4	3	2	22	13
Tuberculide	1	1	0	0	0	100
Scrofulodermia ..	2	2	0	0	0	100
Pulmonary tuberculosis ..	20	16	1	1	2	80
Lupus erythematosus ..	20	1	2	2	15	5
Psoriasis	5	0	1	0	4	0
Tinea cruris	6	0	0	1	5	0
Syphilis	10	1	1	0	8	10

DISCUSSION.

These results indicate that complement-fixation is of little value in the two chief tuberculous diseases of the skin and unfortunately the number of cases of scrofulodermia and tuberculide are too few to indicate whether the high percentage of positive results in these diseases is reliable. Some of the non-tuberculous controls were positive and it is possible that these were caused by non-recognized foci elsewhere in the body. In any case they tend to detract still further from the value of this test in cutaneous tuberculosis.

The presence of active tubercle bacilli is necessary for a positive complement-fixation test and this is evident from the high percentage of positive results found in pulmonary tuberculosis with bacilli in sputum in the majority of cases. So it may be possible that the few positive results found in lupus vulgaris and tuberculosis verrucosa cutis indicate the presence of active bacilli, and the comparatively higher percentage of positive results found in the latter disease may indicate that the bacilli are more numerous and more active in this than in lupus vulgaris. This fact is

borne out in unpublished animal inoculation experiments (rabbits and guinea-pigs) with diseased material in which tuberculosis verrucosa cutis gave a higher percentage of positive results.

SUMMARY AND CONCLUSION.

1.   The complement-fixation test with W. K. K. tuberculosis antigen was performed in 71 tuberculous and 61 non-tuberculous skin diseases.

2.   The results obtained are tabulated and the findings discussed.

3.   This reaction is not of much practical value in diagnosis of cutaneous tuberculosis.

ACKNOWLEDGMENT.

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ON WASSERMANN REACTION.

Part IV*.

THE ANTIGEN.

BY

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In comparing antigens prepared by different procedures one important point emerged. The different lots of the same kind of antigen, prepared according to the same procedure from different hearts, may differ from one another much more than from another kind of antigen. This communication deals with McIntosh and Fildes' antigen (McIntosh, 1931) and Bordet's antigen which with modification has been used in this laboratory for many years. Of the former antigen certain hitherto unstudied aspects, certain aspects concerning which a difference of opinion is possible, and power of fixing complement with titrated controls prepared from especially selected positive sera (Greval, Das and Sen Gupta, 1938) have been studied, means of standardization devised and a new modification described. The

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* For Part III see *Indian Journal of Medical Research*, Vol. **26**, No. 2, 1938, p. 393.

latter antigen has also been standardized. The two antigens have been compared. Observations on fortifying reagents have also been made. A suggestion for using several antigens in a test has been advanced.

I. Plain Alcoholic Heart-Extract, Ingredient 'A' of McIntosh and Fildes' Antigen.

1. *Content.*—The plain alcoholic extract becomes turbid when mixed with saline. A 1 part extract + 14 parts saline suspension prepared from each extract is a very simple means of comparing the contents of the extracts with respect to the alcohol soluble and water insoluble substances. The difference in turbidity may be very well marked at times. It has been found that while extracts yielding poor turbidity are always poor in fixing complement with known syphilitic sera, the extracts yielding good turbidity are not always good. A comparison with a satisfactory extract already in use is sufficient.

The suspension is made by adding, in an ordinary test-tube, to 0·5 c.c. of the extract 7 c.c. of saline *rapidly* and mixing vigorously by hitting the tube against the palm of the hand.

2. *Hæmolytic power.*—The suspension made from the extract is hæmolytic only in high concentration. In a 1 in 15 concentration (1 part extract + 14 parts saline) it should be non-hæmolytic when 1 volume of it is added to 1 volume of a 3 per cent r. b. c. suspension and 2 volumes of saline, otherwise it cannot be used in Wassermann reaction when an unfortified antigen of this strength is employed. As a routine 3 volumes of the suspension are tested with 1 volume of the r. b. c suspension and the extracts which yield hæmolytic suspensions rejected.

3. *Anti-complementary power.*—The suspension 1 in 15 should not be anti-complementary at all when tested with 1 m. h. d. of complement, determined in accordance with method No. 4 of the (British) Medical Research Council (1918, then Committee). If it is, it is rejected. An association has been found between the hæmolytic power and the anti-complementary power. In one sample (out of a total of 23 heart extracts) both were very high. The complement-fixing power of the extract was poor.

4. *Complement-fixing power.*—While it is known that all Wassermann antigens (plain heart extract or ingredient A parts 3 + 1 per cent cholesterol in alcohol or ingredient B parts 2 + normal saline parts 70) are not satisfactory, it is not generally recognized that the usual criterion of a satisfactory antigen is far from adequate. It has been shown in a previous communication (Greval *et al.*, *vide supra*) that the usual 1 in 5 dilution of a known syphilitic serum is utterly useless in comparing new unknown antigens with an old known antigen.

It was found that certain sera in the usual 1 in 5 dilution fixed complement even with a 1 in 15 suspension in saline of the plain extract obtained by pooling six or more extracts. These sera were designated 'strongly positive, + + +'. Further, these sera went on giving the reaction of fixation (full or partial) when diluted 1 in 10, 1 in 25, or 1 in 50. When the + + + sera were collected irrespective of the histories of the cases and from cases from general hospitals the dilution for the reaction tended to lie between 1 in 10 and 1 in 25 ; when they were collected

from venereal clinics and hospitals the dilution tended to lie between 1 in 25 and 1 in 50.. These figures were accepted as representing the limits of reaction of the two divisions of the cases giving a $+ + +$ reaction.

Next was tested each one of the plain extracts with each division of the $+ + +$ sera. Those that gave full fixation (no traces of lysis in the tube after it had stood overnight in the cold) with the serum dilution expected to give full fixation were pooled to yield the standardized heart extract. Others were kept aside for further observations. The first standardized heart extract used had in it five selected heart extracts. Since then the practice has been to pool three selected heart extracts as minimum and six as optimum.

All new extracts are now simply put up, along with the extract in use, with pooled $+ + +$ serum kept for the purpose of providing titrated controls in the daily routine. In the case of a particularly potent serum (from venereal clinics and hospitals) the dilutions used are 1 in 10, 1 in 25, and 1 in 50, and in the case of a moderately potent serum the dilutions are 1 in 10 and 1 in 25 only. Those extracts which compare favourably with the extract in use are selected for pooling and the rest are kept aside for further observations and if possible adjustment.

There has been no occasion to start afresh by pooling a large number of extracts. The new lots of the $+ + +$ serum collected, for the titrated controls in the daily routine, react in accordance with expectations with the old pooled extract, and selection for pooling from the new extracts is made by comparison with the old extract by means of the $+ + +$ serum of known titre.

· 5. *Adjustment of the complement-fixing power.*—Some extracts fix the complement with the known serum to the expected degree not in the usual dilution of 1 in 15 but in stronger dilutions. They are evaporated to concentrate them to the required degree and tested again. Other extracts are found to be ineffective even in stronger dilutions and are kept aside. As a matter of fact for the comparison of the new extract with the old, by means of an old lot of $+ + +$ serum of known titre, the test is put up in two strengths of the extract. The appended ensemble (Table I) explains this procedure.

When the reading of a 1 in 15 dilution of the new extract corresponds to that of a 1 in 15 dilution of the old extract the new extract is selected for pooling. When the 1 in 15 dilution of the new extract does not correspond but a 1 in $12\frac{1}{2}$ dilution does, 15 volumes of the extract are evaporated to yield $12\frac{1}{2}$ volumes and the titration repeated. The reading will now correspond. When the 1 in $12\frac{1}{2}$ dilution does not correspond but gives an appreciably better fixation than the 1 in 15 dilution, the extract is tried in a 1 in 10 dilution. If the latter dilution corresponds 15 volumes of the extract are evaporated to yield 10 volumes and the titration repeated. The reading will now correspond.

When no appreciable difference is found between the fixation given by a 1 in 15 dilution and a 1 in $12\frac{1}{2}$ dilution the extract is not adjustable. It is kept aside for diluting exceptionally sensitive extracts.

The amenability of some extracts to adjustment by concentration and not of others is a proof of a difference of quality as well as of quantity in the heart extracts.

Heart extracts are *selected* with a view to ensuring a high and constant power of fixing complement and *pooled* with a view to eliminating peculiarities of single extracts which might react better with some syphilitic sera than with others.

Heart extracts stored in stoppered bottles and not exposed to light keep indefinitely.

In testing borderline cases and in critical work the standardized antigen is as indispensable as the titrated hæmolytic amboceptor and the titrated complement.

II. Alcoholic solution of cholesterol, ingredient 'B' of McIntosh and Fildes' antigen.

1. *Turbidity and anti-complementary power.*—The usual 1 per cent solution when suspended in saline in the same proportion in which it occurs in a suspension of the mixed antigen (0·1 c.c. sol. + 0·15 c.c. alcohol + 3·5 c.c. saline) is very turbid and strongly anti-complementary. When 2 volumes of it are mixed with 3 volumes of the heart extract, as is done in preparing the standard mixed antigen, these properties suffer a loss yet most of the turbidity and all the anti-complementary power of the mixed antigen are due to cholesterol. When 1 volume of it is mixed with 4 volumes of the extract the anti-complementary power is hardly perceptible.

The anti-complementary power of the mixed antigen is usually 1 m. h. d. of the complement. But it may be, more or less, depending upon the complement. This variation in the complement is so marked and the power of the complement to be fixed depends so much on the variation that the item will be dealt with fully in another communication.

2. *Power of fortifying the heart extract.*—Cholesterol intensifies the antigen inasmuch as it enables the heart extract to react with a weak dilution of a certain class of syphilitic sera (+ + + sera of the writers) and with another class of syphilitic sera which will otherwise be missed altogether (+ + sera of the writers) even in strong dilutions.

3. *Hæmolytic power.*—A suspension of cholesterol, of the same strength in which it occurs in the mixed antigen, when added to r. b. c. suspensions and incubated for ½ hour at 37°C. is not immediately hæmolytic. The r. b. c. which settle down are lysed later, at the bottom of the tube, even in the cold. The tube next day shows a large colourless column of fluid superimposed on a small coloured column of fluid. The vibration of the refrigerator may bring about a certain amount of diffusion yet the distinction is obvious.

The mixed antigen also lyses the r. b. c. in the same way, due to the action of cholesterol. This property of the mixed antigen makes the determination of the end-point of the reaction of a syphilitic serum difficult. Whether the T reaction is due to the commencement of the failure of fixation or to the slow hæmolytic action (and diffusion of colour) of the cholesterinized antigen which has not been all used up by a weak dilution of the syphilitic serum may not be easy to decide.

The hæmolytic action of cholesterol is not universal. In studying the lysability of the r. b. c. of man, monkey, dog, sheep, goat, pig, guinea-pig, and rabbit, under various conditions, it was found that the contact with the mixed antigen, in the proportion in which the r. b. c. and the antigen come into contact in the Wassermann reaction, affects the different cells differently. Dog's corpuscles were most lysable, then came sheep's, while goat's showed only a trace of lysis. Others were not affected. The contact lasted only ½ hour after which the tubes containing the r. b. c. and the mixed antigen were centrifuged and the supernatant fluid replaced by saline.

It appears that goat's blood would be more suitable than sheep's for Wassermann reaction and for other complement-fixation reactions in which cholesterol is used as a fortifying agent. In sheep's blood there is no particular virtue other than its easier availability in Europe and America. A hæmolytic amboceptor against goat's blood can be prepared as easily as against sheep's blood. Some samples of antisheep hæmolytic amboceptor work with goat's r. b. c. quite well. As a laboratory animal a goat is hardier and cheaper than a sheep.

III. The combined antigen.

1. *Mixing of ingredients A and B.*—The usual recommendation is that the heart extract and the cholesterol solution should be kept in separate bottles and mixed immediately before use. The writers' opinion is to the contrary. The cholesterol acting in combination is a much more potent reagent than the heart extract. A slight variation in its measurement, due to a slight inaccuracy in the pipette, in measuring out *small* quantities for the day's work is almost certain to interfere with the uniformity of the fixing power of the suspensions of the combined antigen. *Large* quantities of the heart extract and the cholesterol mixed and used over long periods would be free from this flaw, provided no deterioration occurred with storage. No evidence of deterioration has been found in this laboratory. The combined antigen like the heart extract keeps indefinitely. Sixty c.c. of the extract and 40 c.c. of the cholesterol solution are mixed at a time.

2. *Turbidity of the suspension.*—It is known that a 1 in 15 suspension of the combined antigen in saline is more turbid when the former is superimposed on the latter in a test-tube and the two mixed slowly by rotating the tube, than when the two are mixed quickly. It is also known that the more turbid suspension fixes a little more complement. The gain in fixation, however, is not compensated by the loss of uniformity of turbidity and fixation which occurs when different quantities of the antigen and the saline are mixed for the daily requirements which differ. It is preferred to make the suspension by mixing the antigen and the saline quickly. The required quantities are taken in two test-tubes. The saline is poured into the antigen and the mixture shaken briskly. It is poured into the original saline tube and back again into the original antigen tube. Altogether there are three steps, one mixing and two transfers.

The difference between the fixing power of two suspensions one of which is more turbid than the other is of an order which can lighten the tinge of colour in a titrated ± control. It is not of an order which is likely to convert a ± reaction

into a $+$ reaction in the test proper. Even if it did the advantage would be rejected in favour of the uniformity of the fixing power of the antigen.

3. *The dose.*—The dose of the antigen is in excess of the requirements of the serum. A further dilution of a 1 in 15 suspension to a 1 in 30 suspension does not reduce appreciably its power of fixing complement and strange though it may seem not even its anti-complementary power although its turbidity is definitely reduced. Its late lytic action on r. b. c. is also reduced.

4. *Abolition of the late lytic action on r. b. c. in the test proper.*—Addition of a 1 in 5 dilution of the serum to the antigen suspension such as occurs in the test proper abolishes the lytic action on r. b. c. It is immaterial whether the serum is positive or negative.

5. *Reaction with negative sera.*—Every new stock of combined antigen is tested with six negative sera left over from the previous day's work, as a precautionary measure. It has never been found to give a positive or a doubtful reaction.

IV. Phenol as a fortifying agent.

0·25 per cent phenol added to either the serum, the heart-extract suspension or the combined antigen suspension enhances the fixation of the complement appreciably. The percentage of the phenol in the total quantity of the fluid is $\frac{0\cdot25}{4} = 0\cdot0625$.

Further, phenolization of the suspension of the combined antigen also abolishes its late lytic action. In this connection it is of interest to note that :—

1. Heart extract is hæmolytic.

2. Cholesterol is also hæmolytic ; but heart extract and cholesterol are less hæmolytic together than separately.

3. Phenol is also hæmolytic ; but heart extract, cholesterol, and phenol together are less hæmolytic than separately.

0·25 per cent phenol added to the antigen suspension does not interfere with the m. h. d. of the complement determined in the presence of the antigen.

Advantage can be taken of the fortifying action of the phenol when the amount of cholesterol in the combined antigen has to be reduced or when the combined antigen does not react to the expected degree. This adjustment and occurrence are consequent on variation in complement.

Lately, trikresol (Schering-Kahlbaum) has been used instead of phenol (B.P.) in preserving or fortifying reagents in complement-fixation. It appears to be more stable so far as in saline clarity of solution and absence of pink colour, and in serum dilution appearance of precipitate are concerned.

V. Bordet's antigen.

1. *Preparation and use.*—This antigen, which has been in use in this laboratory for a long time, is prepared as follows :—

(1) One hundred* grammes of human heart muscle cut into pieces (not minced) are left in contact with 125 c.c. of absolute alcohol, in a stoppered bottle, for a week.

(2) The alcohol is poured off and the muscle dried in an incubator for 24 to 48 hours (should be like pieces of wood).

(3) The dried muscle is left in 200 c.c. acetone for a week, in a stoppered bottle.

(4) The acetone is poured off and another lot of 200 c.c. added.

(5) After 24 hours the second lot of acetone is also poured off and the muscle dried in an incubator until free from smell (3 days or more).

(6) The dried muscle is powdered and the powder extracted with 200 c.c. of absolute alcohol for 10 days, in a stoppered bottle.

(7) The stoppered bottle is shaken and the contents filtered through a filter-paper. The filtrate is Bordet's antigen.

(8) To 30 c.c. of Bordet's antigen are added 20 c.c. of 1 per cent of cholesterol in absolute alcohol. The mixture is cholesterinized Bordet's antigen.

Of the finished product a 1 in 50 suspension is made in saline and used in the place of the other antigen.

In the original technique the relative quantities of the antigen, the serum and the hæmolytic system differed slightly from the quantities employed in Method IV. The 1 in 50 dilution was arrived at by calculation and has finally been adopted after trial and obtaining agreement with the results obtained by the original technique, in over 1,000 consecutive tests, on observed cases, during one year.

2. *Advantage and disadvantages.*—The suspension is not anti-complementary. Therein lies its advantage over the suspension of the other antigen. No titration of the complement in the presence of the antigen is required.

The fixation of the complement effected by the suspension varies with the type of the complement, because of the cholesterol contained in it, exactly as occurs in the case of the suspension of McIntosh and Fildes' antigen. The titrated controls show similar increase and decrease in each set. The type of the complement is determined by the anti-complementary titre of McIntosh and Fildes' antigen. In working with Bordet's antigen alone no such determination is possible. The necessary adjustments in the antigen-complement system cannot be made. Herein lies a serious disadvantage of Bordet's antigen.

The fixation effected by Bordet's antigen is on the whole slightly higher. The titrated controls + and ± adjusted for McIntosh and Fildes' antigen more often than not fail to show the difference with Bordet's antigen. Considering that the titrated controls + and ± are generally 1 in 50 and 1 in 100 dilutions, respectively, of a strongly positive serum (+ + +) the difference should be shown. A failure

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* This and the corresponding quantities used in extraction are actually halved. From the same heart 50 grammes are used for Bordet's antigen and 10 grammes for McIntosh and Fildes' antigen.

in this respect is more of a defect than of a virtue indicative of a higher sensitiveness. Clinically too, Bordet's antigen is less helpful in differentiating between early, late and treated cases.

3. *Selection.*—Like the plain alcoholic extract antigen Bordet's antigen should also be selected and standardized. The two antigens were prepared from the same heart and found to correspond very closely. Now, when the plain extract from a heart is found unsatisfactory without adjustment the preparation of the other extract from the same heart is not proceeded with. In the writers' procedure this antigen is used in one tube out of a total of four. Its stocks, consequently, last longer.

VI. A COMPARISON OF THE REACTIONS OF TWO SERIES WITH THREE ANTIGENS, AND ASSOCIATED CONSIDERATIONS.

1. *The reactions.*—In the appended table (Table II) are given 2,000 reactions from 2,000 sera, received mostly from the hospitals in Calcutta, with (*i*) alcoholic heart extract uncholesterinized antigen, for $+ + +$ reaction, (*ii*) alcoholic heart extract cholesterinized antigen, for other reactions, and (*iii*) Bordet's antigen cholesterinized and hereafter called Bordet's antigen, one tube only. The two series which represent work done on 31 working days are given separately to bring out the variations in two otherwise similar batches.

2. *Explanations.*—The positive rate of the table is *not* the positive rate of the unselected Calcutta hospital population. More than half of the cases tested are syphilitic suspects. In them a high positive rate is expected. Only less than half, from a small hospital population, are tested as a routine. The positive rate for the latter population in Calcutta, as calculated by Greval, Sen Gupta and Das (1938), is *under* 10 per cent.

Included in $+ + +$ reactions in the series are some $+ + T$ (a trace of lysis instead of the third $+$) reactions, *just* $+ + +$. Also included in $+ +$ reactions are some $+ T$ reactions, *just* $+ +$. $+ \pm$ reactions have been classed with $+$ reactions of which, in fact, they constitute a major part. Plain $+$ (i.e., $+ -$) reactions are always rare. TT reactions are classed with $\pm \pm$ and $\pm -$ reactions, doubtful. ? $-$ and $-$ are classed together.

Percentage of $+ + +$ reactions is high in both the series and is derived from syphilitic suspects and from cases from venereal hospitals and clinics. When the blood is tested as a routine from the general wards of hospitals it is low.

The $+ + +$ sera would be positive with any antigen and by any technique one chooses to employ. Agreement between the reactions of such sera with different antigens, therefore, is not of much value. For all practical purposes, then, the comparison lies between the reactions of the other sera with (*i*) cholesterinized alcoholic heart-extract antigen and (*ii*) Bordet's antigen.

The number of anti-complementary sera is given at the end of each series in brackets. The total number of cases taken from the register is 2,007. Twelve C.S.F.'s are also included in the total.

3. *Agreement and disagreement.*—The absolute agreement and the relative agreement, taken together, are good. The total disagreement is small.

It will be observed that (*i*) the positive rate with Bordet's antigen of the two series is almost identical, (*ii*) the positive rate with the cholesterinized heart-extract antigen varies, (*iii*) the negative rate of the two series is of about the same order, the number of negative cases with the two antigens in the first series being almost identical, and that (*iv*) the number of + + + cases, with the uncholesterinized heart-extract antigen, is almost identical in the two series.

4. *Does a positive reaction with Bordet's antigen increase and a negative reaction decrease the value of a positive reaction with the other antigen ?*—From a consideration of the figures, of the reaction of the titrated controls and of the reaction of specially observed cases, the answer is, no. Bordet's antigen is, on the whole, more *sensitive* but not more *specific*. In certain tropical and non-tropical morbid states, other than syphilis, both the antigens give false positive reactions with equal frequency. In some cases of tertiary syphilis, clinically unmistakable, Bordet's antigen has given negative reactions. Even the reaction of some lots of pooled + + + sera, diluted and used for titrated controls, has weakened, with the age of the serum, with Bordet's antigen and not with the other antigen.

5. *Does a positive reaction with Bordet's antigen cast a doubt on a negative reaction with the other antigen ?*—The writers have now come to the conclusion that there is no harm in the presumption that it does. Such cases are read as doubtful (±) and the clinical details studied. If the details are suggestive the reaction is returned as doubtful, otherwise it is returned as negative and a repetition of the test is suggested, in two weeks, without treatment or provocative injections. Incidentally the observations of the writers on the value of the provocative injection are not for the injection. Cases are also returned as doubtful when they give a T reaction with Bordet's antigen and a ? − reaction with the other antigen. T and ± reactions with Bordet's antigen are rejected if they contradict a clear-cut negative reaction with the other antigen.

6. *Is a double negative reaction with the two antigens more significant than a single negative reaction with the ordinary cholesterinized alcoholic extract antigen accompanied by a doubtful reaction with Bordet's antigen ?*—From a consideration of the figures alone the answer is, yes.

7. *Is the significance of a doubtful reaction with the ordinary antigen increased when it is accompanied by a positive reaction with Bordet's antigen ?*—The answer is, yes. But still it will be a doubtful reaction (±) for the purpose of the report. A positive result should not be based on the reaction with Bordet's antigen which has not been tried with the normal cases and the known cases of syphilis, to the same extent to which the other antigen has been tried. Lloyd (1932) from this laboratory wrote : ' It is known that antigen (3) (cholesterinized Bordet's antigen— S. D. S. G.) is frequently too sensitive for use where an unknown serum is being examined for a diagnosis, and cases at times occur in which this antigen yields a positive, while the other two yield a negative result. Should such a result be obtained the case would be reported negative if nothing were known about it.'

8. *Is the significance of a doubtful reaction with the ordinary antigen decreased when it is accompanied by a clear-cut negative reaction with Bordet's antigen ?*—The

writers are inclined to believe that, in a case suspected clinically to be an early case of syphilis, it is. Such cases will continue to be returned as doubtful, however, until a sufficiently large series has made this belief absolute.

VII. McIntosh and Fildes' antigen phenolized.

A 1 in 15 suspension of the ordinary combined antigen in saline containing 0·25 per cent phenol (trikresol) in its power of fixing complement approaches Bordet's antigen very closely. In fact it has two advantages over the latter, (i) it is always stronger than the ordinary antigen, while Bordet's antigen may at times prove weaker, and (ii) it nearly always differentiates between a + and a ± titrated control. The phenol in it does not interfere with the m. h. d. of the complement.

This antigen is recommended for detecting disappearing reactions of previously positive treated cases. The increase in fixation of complement is of an order which will convert a ? — into a ±, a ± into a T and a T into a +.

In a future communication will be described another use of this antigen, in reading the result of fixation, with a particular type of complement.

VIII. Antigens made from other than human hearts.

Animals of 'guinea-pig type' (Browning, 1931), i.e., guinea-pig, horse, dog, cat, mouse, fowl, and tortoise, have in their organs Forssman's antigen. This antigen is alcohol soluble and reacts with the heterophile antibody which can only occur in animals of 'rabbit type', i.e., rabbit, man, ox, sheep, rat, goose, pigeon, eel and frog. That the antibody may occur as a natural antibody in some human subjects is a possibility. This consideration has led the writers to exclude hearts of the animals of the guinea-pig type.

Antigens made from beef, goats' or sheep's hearts have been used. Beef hearts have yielded poor, goats' hearts moderately good, and sheep's hearts good extracts. In fact more sheep's heart extracts have come up to the standard in selection than have done the human heart extracts. These remarks apply to both the plain alcoholic extracts and the extracts prepared after a preliminary extraction of the heart with acetone.

IX. Use of several antigens in a test : a suggestion.

1. *Testing of sera.*—Three antigens are used in testing each serum :—

   (i) Standardized (as described in this communication) McIntosh and Fildes' antigen used in accordance with the requirements of Method IV of the (British) Medical Research Council, i.e., with 3 and 5 m. h. d. of complement. The reaction of the two tubes are recorded and reported as detailed in a previous communication (Greval, Das and Sen Gupta, 1938).

(*ii*) Standardized plain alcoholic heart extract with 4 m. h. d. of complement. This dose of the complement functionally equals 5 m. h. d. used with the ordinary antigen which is anti-complementary to the extent of 1 m. h. d. The positive reaction of this tube is read + + +, strongly positive. The two tubes of the ordinary antigens are also positive in this case.

(*iii*) Standardized Bordet's antigen with 2 m. h. d. of complement. This dose of the complement functionally equals 3 m. h. d. used with the ordinary antigen.

Ordinary antigen phenolized can replace Bordet's antigen.

2. *Testing of cerebro-spinal fluids.*—Partly following Wyler (1929). Cerebro-spinal fluid, without inactivation, is also put up with the three antigens in two strengths : (*i*) undiluted fluid and (*ii*) two volumes of undiluted fluid instead of one volume. 5-m. h. d. tube is omitted. Inhibition of lysis in the tube containing two volumes of the undiluted cerebro-spinal fluid, even when complete, is not accepted as a positive reaction unless there is also a definite inhibition of lysis in the tube containing one volume of undiluted fluid. The results are read :—

+ + + = Strongly positive, when complete inhibition of lysis occurs with the plain heart extract in one or both tubes, subject to the provision concerning two volumes.

+ + = Positive, when complete inhibition occurs with the ordinary antigen, in both tubes.

+ = Weakly positive, when inhibition is complete with the ordinary antigen with 2 volumes of the fluid and partial but well marked with 1 volume.

± = Doubtful, when partial but well-marked inhibition occurs in one or both tubes with ordinary antigen.

— = Negative, when no inhibition or inhibition of a poor quality occurs with the ordinary antigen.

The tube put up with Bordet's cholesterinized antigen supports the inhibition of lysis in the corresponding tube put up with the ordinary antigen.

Ordinary antigen phenolized can replace Bordet's antigen.

3. *Several antigens in Wassermann reaction versus several tests for syphilis.*— The suggestion is advanced that a Wassermann reaction done with several antigens should be preferred to several different reactions done for syphilis. When all is said and done the flocculation tests have not succeeded in taking the place of the Wassermann reaction. Harrison (1931) ' would not found a diagnosis on a serum test unless the Wassermann reaction was positive '. This statement typifies the opinion of most workers even after the issue of the League of Nations' Health Organization Report of the Second Laboratory Conference on the Sero-Diagnosis of Syphilis (League of Nations' Health Organization, 1928), resolution I of which states that the conference ' ....is of opinion that the best of them (flocculation tests—S. D. S. G.) may be regarded as equal in value to the best of those which depend on fixation of complement (Bordet-Wassermann) '.

TABLE I.

An ensemble showing the titration of a new heart extract.

Sets of tubes.	A set with old heart extract, 1 in 15.			A set with new heart extract, 1 in 15.			A set with new heart extract, 1 in 12½.		
Known +++ serum, 1 vol. of 1 in :—	10	25	50	10	25	50	10	25	50
Complement, 4 m. h. d.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.
Heart extract (old 1 in 15, new 1 in 15 or new 1 in 12½).	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.
	Incubated at room temperature ¼ hour and at 37°C. ½ hour.								
Sensitized and standardized r. b. c.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.
	Incubated at 37°C. for ½ hour.								
	Left in the cold overnight.								

Results for each set are read as :—

        + + + = Complete inhibition of lysis in the three tubes of a set.
        + + T = A trace of lysis in the last tube.
        + + ± = Incomplete lysis in the last tube.
          + + = Complete inhibition in two tubes only.
          + T = A trace of lysis in the second tube.
          + ± = Incomplete lysis in the second tube.
            + = Complete inhibition in one tube only.
            T = A trace of lysis in the first tube.
            ± = Incomplete lysis in the first tube.

TABLE II.

Comparison of reactions of 2,000 cases with uncholesterinized alcoholic heart-extract antigen, cholesterinized alcoholic heart-extract antigen, and cholesterinized Bordet's antigen.

Series 1.   1,000 cases tested from 11th April, 1939 to 8th May, 1939 (on 15 working days).

	CATEGORIES.		
	Reaction with alcoholic heart extract, uncholesterinized (for +++) and cholesterinized (for other reactions).	Reaction with cholesterinized Bordet's antigen (one tube).	Number of cases.
I ..	+++	+	55
II ..	++	+	65
III ..	++	±	8
IV ..	++	—	4
V ..	+	+	10
VI ..	+	±	3
VII ..	+	—	1
VIII ..	±	+	68
IX ..	±	±	44
X ..	±	—	49
XI ..	—	+	22
XII ..	—	±	34
XIII ..	—	—	637
		(includes 4 C.S.F.)	
		TOTAL ..	1,000
(XIV ..	Anti-complementary, includes 1 C.S.F.		1)

Change in reaction summarized to show absolute agreement, relative agreement, and total disagreement.

Reactions with alcoholic extract. | Reactions with Bordet's antigen.

				+	±	—
+ (all grades)	..	146	=	130	11	5
±	..	..	161	=	68	44	49
—	..	..	693	=	22	34	637
		1,000				

Reactions with Bordet's antigen. | Reactions with alcoholic extract.

				+	±	—
+	..	..	220	=	130	68	22
±	..	..	89	=	11	44	34
—	..	..	691	=	5	49	637
		1,000				

TABLE II—*concld.*

Series 2.   1,000 cases tested from 9th May, 1939 to 3rd June, 1939 (on 16 working days).

	CATEGORIES.		
	Reaction with alcoholic heart extract, uncholesterinized (for +++) and cholesterinized (for other reactions).	Reaction with cholesterinized Bordet's antigen (one tube).	Number of cases.
I	+++	+	57
II	++	+	96
III	++	±	17
IV	++	—	5
V	+	+	20
VI	+	±	7
VII	+	—	2
VIII	±	+	33
IX	±	±	50
X	±	—	57
XI	—	+	13
XII	—	±	37
XIII	—	— (includes 7 C.S.F.)	606
		TOTAL ..	1,000
(XIV	Anti-complementary		6)

Change in reaction summarized to show absolute agreement, relative agreement, and total disagreement.

Reactions with alcoholic extract.			Reactions with Bordet's antigen.		
			+	±	—
+ (all grades) ..	204	=	173	24	7
± ..	140	=	33	50	57
— ..	656	=	13	37	606
	1,000				

Reactions with Bordet's antigen.			Reactions with alcoholic extract.		
			+	±	—
+ ..	219	=	173	33	13
± ..	111	=	24	50	37
— ..	670	=	7	57	606
	1,000				

SUMMARY.

1. Different lots of Wassermann antigens, prepared according to the same procedure, from different hearts, may differ from one another much more than from another kind of antigen prepared according to another procedure.

2. Plain alcoholic heart extract can be selected and standardized at this stage, by comparing its power of fixing complement, with a certain dilution of a pooled + + + serum, with that of a known extract found to be satisfactory. Details are given. For borderline cases and critical work the standardized antigen is indispensable.

3. Hæmolytic, anti-complementary. and fortifying actions of cholesterol have been studied. The hæmolytic and anti-complementary action of the combined antigen is due to cholesterol.

4. The plain alcoholic heart extract and the cholesterol solution should be mixed in large quantities and stored as combined antigen. The suspension from the latter should be made by sudden dilution with saline.

5. Phenol is also a fortifying agent. It abolishes the hæmolytic action of the combined antigen.

6. Preparation of Bordet's antigen, as used in this laboratory for a long time, has been described in detail. It should also be selected and standardized.

7. Two thousand reactions with three antigens (plain alcoholic heart-extract, cholesterinized plain alcoholic heart-extract, and cholesterinized Bordet's antigen) have been tabulated, and explanations and conclusions given.

8. Cholesterinized plain alcoholic heart-extract antigen (the ordinary combined antigen) can be phenolized and made to approach Bordet's antigen in sensitiveness. It has definite uses.

9. Good antigens can be prepared from sheep's hearts.

10. A suggestion is advanced that a Wassermann reaction done with several antigens should be preferred to several different reactions done for syphilis.

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HÆMATOLOGICAL STUDIES IN INDIANS.

Part XI.

THE CHOICE OF AN ANTI-COAGULANT*.

BY

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SOKHEY *et al.* (1937) drew attention to the discrepancies in the literature regarding the percentage of shrinkage that occurred in the red cell volume when potassium oxalate, 2 mg. per c.c. of blood, was added to prevent coagulation. Various workers gave figures that varied from 3·5 per cent to 8·25 per cent shrinkage. These writers, using the same percentage of potassium oxalate, obtained a figure of 5·75 per cent for shrinkage.

They also drew attention to the present writers' observations in this connection. We had in two small series used two isotonic anti-coagulants—heparin and hirudin—as standards, and had obtained with 0·2 per cent potassium oxalate mean shrinkages of 8·07 and 6·86 per cent respectively, in the two series, or, if the findings were taken together, the mean shrinkage was 7·35 per cent (wrongly calculated by us as 7·94 per cent).

Sokhey *et al.* (*loc. cit.*) expressed doubts about the propriety of calculating an average from so widely differing ranges of values, doubts which we ourselves shared with them and which had led us to ignore our own figure and accept Wintrobe's most recent figure of 8·2 per cent shrinkage.

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* The work reported here was done 18 months ago, but we put our findings aside as we felt that further work was necessary. We did not pursue the investigation further and, as we now feel that it is unnecessary to do so, we are reporting these findings.

It is true that Wintrobe (1932) does not explain the discrepancies in his findings nor state on how many cases or estimations he based this figure, but it is the identical figure that Haden (1930) obtained with the same strength of the same anti-coagulant and the one that is now generally accepted by both British (Whitby and Britton, 1937) and American hæmatologists.

Early in 1937, Wintrobe in a personal communication to one of us (L. E. N.) reported that he had found a mixture of potassium and ammonium oxalate, 4 mg. and 6 mg. respectively, added to 5 c.c. of blood, formed an isotonic solution. We have since found that he has described this method (Wintrobe and Landsberg, 1935).

We carried out duplicate packed-cell-volume estimations in a series of 50 specimens of blood taken from various sources, using as coagulants potassium oxalate 2 mg. to 1 c.c. blood in one side of the centrifuge and the ammonium and potassium-oxalate mixture in the other*. The speed of the centrifuge was about 3,000 revolutions per minute and the time was 30 minutes.

The data are given in the table below :—

TABLE.

Cell volume.

Number.	Potassium oxalate 0·2 per cent.		Ammon. and potassium-oxal. mixture. (B)	Differences B—A.
	Crude.	Corrected (A).		
1	32·7	35·64	35·10	−0·54
2	15·1	16·51	16·58	+0·07
3	37·0	40·37	39·80	−0·57
4	29·2	31·78	32·00	+0·22
5	25·0	27·25	27·00	−0·25
6	37·5	40·87	39·00	−1·87
7	25·0	27·25	27·25	0
8	23·8	25·89	25·00	−0·89
9	24·0	26·16	26·25	+0·09

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* For the collection of blood, it is convenient to have small flasks ready containing the requisite amount of oxalate powder. The amount of oxalate is small, so dilute aqueous solutions of potassium and ammonium oxalate are made and the requisite amounts of these are put into each flask with a graduated pipette. The flasks are then dried in a hot-air oven when the oxalate mixture will be left in the flask in the form of powder.

For each c.c. of blood 0·4 c c. of 0·2 per cent potassium-oxalate solution + 0·6 c.c. of 0·2 per cent ammonium-oxalate solution is required.

TABLE—*contd.*

Number.	POTASSIUM OXALATE 0·2 PER CENT.		Ammon. and potassium-oxal. mixture (B).	Differences B—A.
	Crude.	Corrected (A).		
10	35·0	38·15	38·16	+0·01
11	30·8	33·57	33·66	+0·09
12	28·0	30·50	30·00	−0·50
13	38·0	41·42	40·90	−0·52
14	13·8	15·06	15·35	+0·29
15	28·8	31·34	30·61	−0·73
16	19·0	20·71	20·50	−0·21
17	33·7	36·73	36·80	+0·07
18	24·5	26·70	26·50	−0·20
19	27·7	30·20	30·00	−0·20
20	27·0	29·40	30·00	+0·60
21	26·0	28·30	27·00	−1·30
22	26·0	28·30	27·80	−0·50
23	27·8	30·30	30·00	−0·30
24	30·5	33·20	33·20	0
25	32·9	35·90	36·00	+0·10
26	16·2	17·66	18·00	+0·34
27	24·0	26·16	27·00	+0·84
28	30·7	33·50	34·00	+0·50
29	30·5	33·20	32·10	−1·10
30	23·9	26·00	27·25	+1·25
31	22·0	24·00	24·50	+0·50
32	40·4	44·00	44·70	+0·70
33	42·2	46·00	45·70	−0·30
34	27·5	30·00	30·50	+0·50

TABLE—*concld.*

Number.	POTASSIUM OXALATE 0·2 PER CENT.		Ammon. and potassium-oxal. mixture (B).	Differences B—A.
	Crude.	Corrected (A).		
35	29·4	32·00	31·60	—0·40
36	32·1	35·00	35·70	+0·70
37	43·0	46·90	45·20	—1·70
38	45·8	49·90	48·20	—1·70
39	30·0	32·70	32·25	—0·45
40	37·0	40·30	41·00	+0·70
41	38·4	41·90	42·00	+0·10
42	23·0	25·10	25·00	—0·10
43	8·0	8·70	8·90	+0·20
44	30·0	32·70	31·75	—0·95
45	17·0	18·50	18·75	+0·25
46	33·2	36·20	35·00	—1·20
47	22·6	24·60	26·00	+1·40
48	22·0	23·98	23·50	—0·48
49	28·0	30·50	31·50	+1·00
50	19·3	21·00	22·50	+1·50
TOTAL ..	1,415·0	1,542·00	1,537·06	..
Mean ..	28·300	30·8400	30·7412	..

It will be seen that the mean of the 'corrected' volume of the potassium samples is almost the same as that of the mean volume of the samples with the isotonic anti-coagulant. The difference in the individual estimation is usually less than 1 per cent but is as high as 1·87 per cent in one instance. These differences probably can be accounted for in the reading of the percentages. (It is only possible to read to the nearest 1 per cent or possibly 0·5 per cent but in practice the tubes are not always filled accurately to the 100 mark and when the final reading is taken a correction for this fact has to be made.)

These observations appear to corroborate Wintrobe's findings. If this ammonium and potassium mixture is an isotonic mixture, then his estimate of 8·2 per cent shrinkage appears to have been the right one for 0·2 per cent potassium oxalate, and conversely, if his correction of × 1·09 for 0·2 per cent potassium oxalate, a correction which has been confirmed by others and is almost universally accepted, is the right one, then our figures indicate that his ammonium and potassium-oxalate mixture forms an isotonic solution in which no shrinkage takes place.

Whilst we agree with Sokhey and his co-workers that it is better to give findings uncorrected rather than apply a correction about which one is uncertain, it is not satisfactory to calculate mean corpuscular volumes, etc., on the basis of cell volumes that may be too small by as much as 8 per cent and it would be much more satisfactory if all workers used the same anti-coagulant. Wintrobe's mixture (ammonium oxalate 6 mg. and potassium oxalate 4 mg. to 5 c.c. of blood) appears to be a satisfactory one and has the great advantage in being much cheaper than either heparin or hirudin.

SUMMARY.

Packed-cell-volume estimations were done in duplicate on 50 samples of human blood from various sources. In one side of the centrifuge 2 mg. of dry potassium oxalate were added as anti-coagulant to each c.c. of blood and in the other a mixture of 0·8 mg. of potassium and 1·2 mg. of ammonium oxalate. The results are tabulated.

If Wintrobe's factor, × 1·09, to compensate for shrinkage due to potassium oxalate be applied in the case of the former, the means of the two series are almost identical.

It is suggested that this potassium and ammonium-oxalate mixture be adopted as a standard anti-coagulant.

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A NOTE ON THE BIOCHEMICAL AND SEROLOGICAL CLASSIFICATION OF THE *STAPHYLOCOCCI*.

BY

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AND

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THE classification of the *Staphylococci* presents certain difficulties for, although fairly satisfactory methods exist for distinguishing the pathogenic from the saprophytic varieties, methods of differentiating the various types of pathogens have not been fully worked out.

In view of the number of recent papers that have appeared in Europe and America on the serological and biochemical classification of pathogenic staphylococci it was decided to try certain of these methods to see whether they were applicable to organisms isolated in India. Strains were obtained from patients at the Amritsar Civil Hospital through the courtesy of Major S. M. K. Mallick, I.M.S., and were also isolated locally at Kasauli.

MATERIAL AND METHODS.

Isolation of strains.

In the hospitals supplying the specimens, pus or other infected material was smeared on blood-agar slopes, which were forwarded to Kasauli where they were plated out on nutrient agar and blood agar, a typical colony was then picked off and further plated after having been identified as a *Staphylococcus* by microscopic examination. Similar methods were employed in the case of organisms from rubbings of presumably healthy skin of inhabitants of Kasauli with the object of obtaining strains which might be of non-pathogenic type, for comparison; all the strains dealt with in this report were from human sources.

The following points of each strain were studied :—

    (*a*) Pigment production.
    (*b*) Gelatine liquefaction.
    (*c*) Mannite fermentation.

(d) Hæmolysin production.

(e) Coagulase test.

(f) Serological investigations.

(a) *Pigment production.*

Pure cultures were grown on agar at 37°C. for 24 hours and left at room temperature for a month. The colour obtained varied from white to deep golden-yellow but it was found impossible to differentiate between the gold and yellow described by previous workers as the grades of colour obtained presented far too many intermediate shades. Thus, for the purpose of this paper the strains are only described as pigmented or white, although one might have differentiated some as golden (*aureus*) or yellow (*citreus*).

(b) *Gelatine liquefaction.*

Stab cultures in 15 per cent gelatine were made and kept at 22°C. for a month and liquefaction noted.

(c) *Mannite fermentation.*

The strains were grown in peptone water containing one per cent mannitol and Andrade's indicator. The majority of the strains producing acid did so after a few days' incubation but no strain was reported as negative until incubation had been continued for sixteen days.

(d) *Hæmolysin production.*

The *Staphylococcal* strains were grown on a semi-solid agar medium containing 1 per cent Lab-Lemco, 4 per cent peptone (Witte's), and 0·6 per cent agar buffered at pH 7·0 with a M/15 phosphate buffer. The cultures were incubated in an atmosphere of 20 per cent carbon-dioxide for six days, and the medium was filtered through gauze and tested for the presence of hæmolysins against rabbits' and sheep's cells. In all but three cases where hæmolysin production is shown as positive the α-hæmolysis, i.e., of rabbit cells, proceeded further than the hæmolysis of sheep cells. In the three cases, the β-hæmolysis, i.e., hot-cold hæmolysis of the sheep cells, occurred at considerably higher dilutions of the toxin than did the α-hæmolysis of rabbit cells.

(e) *Coagulase test.*

The coagulase test was carried out by a modification of the method described by Thompson and Khorazo (1937), i.e., oxalated rabbit plasma was obtained by adding 10 c.c. of blood to the residue obtained by evaporating to dryness in an incubator 0·25 c.c. of sterile 8 per cent potassium-oxalate solution. To 0·5 c.c. of this plasma a similar volume of a 24-hour broth culture of the *Staphylococcus* under investigation was added and the mixture incubated at 37°C. for 4 hours. A positive test was indicated by solidification of the mixture.

(f) *Serological investigations.*

Preparation of agglutinating sera.—The method employed for serological investigation of the various strains was that described by Cowan (1939) and for this

purpose three of his type strains were kindly provided by him. Each *Staphylococcal* type strain was grown in ordinary broth for 18 hours and killed by heating at 60°C. for one hour. The suspension was then centrifuged, the supernatant fluid discarded, and the mixture brought to the original volume with normal saline. One c.c. of this suspension was injected intravenously into rabbits on three consecutive days, the procedure being repeated on three separate occasions at weekly intervals. The rabbits were bled on the 7th and the 9th day after the last inoculation.

Absorption of sera.—To obtain pure-type sera the sera prepared as above were absorbed by incubating together 1 c.c. of the original serum with 2 c.c. of a thick saline suspension of agar cultures of each of the other two types of organisms for two hours in the incubator and in the refrigerator overnight, the resulting centrifuged serum thus being 1 in 5 dilution of the original.

Agglutination.—The organism to be tested was grown for 18 hours in ordinary broth, rapidly heated in a water-bath at 100°C. for half an hour, cooled, centrifuged, and the deposit re-suspended in 0·5 c.c. broth. One drop of this suspension from a fine capillary pipette was mixed on a slide with a loopful of the serum diluted 1 in 5. The slide was rocked for three minutes and agglutination observed by naked-eye and hand-lens examination. In those cases where agglutination was observed with more than one of the type sera the test was repeated with absorbed sera. In addition to these strains that fell into definite serological groups, certain strains are reported as unstable, others as giving irregular agglutination, and lastly a small group was observed in which no agglutination occurred with any of the sera.

RESULTS.

The results obtained in the various tests are shown in the tables. Table I shows the biochemical and serological results obtained with the strains isolated from pathological sources and from the smaller number of strains-isolated from the skin of healthy persons. As explained above the strains under investigation were first plated out, identified as *Staphylococci*, and all the tests carried out on a pure culture of a single colony. With most organisms such a procedure would produce satisfactory results, but unfortunately, with such an ubiquitous group of organisms as the *Staphylococci*, complications are introduced as it is possible to find, or accidentally contaminate, a pathogenic staphylococcal specimen with a non-pathogenic organism, and similarly pathogenic organisms may often be found existing saprophytically on healthy skin. It is of course impossible to determine whether any particular organism is pathogenic by animal inoculation as those strains that produce lesions in laboratory animals are not necessarily harmful to man.

Examination of Table I, however, brings out certain definite points. It can be seen, for example, that 83 per cent of the strains from pathological sources are pigmented compared with the 18 per cent of non-pathogenic origin. That non-chromogenic strains are sometimes pathogenic is of course well known, in fact the classical toxin-producing strain (Wood 86) is non-pigmented, and the usual assumption is that the pathogenic white strains are variants from the pigmented strains (Blair, 1939). However, these findings again demonstrate the unreliability of the classical classification into *aureus* and *albus* groups from the point of view of pathogenicity, and therefore in Table II where the organisms are shown classified according to colour the *albus* group is divided into two parts: (*a*) the pathological and

(*b*) the saprophytic in the same way as in the table given by Cruickshank (1937), in this case the potential pathogenicity of the organism being judged by the presence of a positive coagulase test, a finding which most observers (Blair, *loc. cit.*; Fisher, 1936) consider runs parallel with pathogenicity. Further examination of Table I shows that though the organisms from pathogenic sources gave a higher proportion of gelatine liquefiers, mannite fermenters, and hæmolysin-producing strains, these characteristics were not constant enough to distinguish the types under examination.

Table III was prepared to compare the biochemical and serological characteristics of the coagulase-positive and coagulase-negative strains, because of the very close relationship that has been claimed to exist between pathogenicity and a coagulase-positive result (Blair; Fisher; *loc. cit.*). If the production of coagulase is taken as the criterion for a pathogenic strain a much closer correlation is shown in the results of the other tests, viz., positive gelatine liquefaction, mannite fermentation, and hæmolysin production in the case of coagulase-producing strains than in the case of coagulase-non-producing strains.

The results obtained in the agglutination tests are shown in the tables. The most striking point is that only 33 per cent of the strains from pathological sources (Table I) gave definite agglutination with sera of Cowan's three types, and even if a positive coagulase reaction is considered a better criterion of pathogenicity Table III shows that the figure is only improved to 69 per cent. This figure is exactly the same as that obtained by Cowan (*loc. cit.*), but here we also obtained no less than 18 per cent type-positive strains in the organisms from non-pathological sources or 29 per cent in the coagulase-negative group compared with Cowan's zero findings.

SUMMARY AND CONCLUSIONS.

In the examination of 101 strains of *Staphylococci* isolated in India, from pathological sources and healthy skins, the following points were collected :—

1. The majority of the strains (76 per cent) isolated from pathological sources was pigmented and in addition gave a positive coagulase test (*see* Table IV).

2. The majority (76 per cent) from healthy skin was non-pigmented and did not produce coagulase.

3. An equal proportion from pathological sources and healthy skins gave a positive result with one or other of these tests.

4. 90·5 per cent of the *Staphylococci* from pathological sources were either pigmented or coagulase-positive.

5. The application of serological classification on the lines adopted by Cowan (*loc. cit.*) resulted in the classification of only 33 per cent of the strains from pathological sources, while no less than 18 per cent of strains from healthy skins gave agglutination with his serological types.

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TABLE I.

Staphylococcal strains classified on source.

		Pigmented.	Gelatine.	Mannite.	Haemolysis.	Coagulase.	SEROLOGY.			Type.		
							Unstable.	Negative.	Atypical.	1	2	3
Pathological source 84.												
		70	73	72	67	70	7	3	19	3	10	14
Percentage	..	83	87	86	80	83	8·3	3·6	23	3·6	12	17
Non-pathogenic source 17.												
		3	12	5	6	3	2	8	4	2	1	0
Percentage	..	18	71	30	35	17	12	47	23	12	6	0
Totals 101.												
Number and per cent		73	85	77	73	73	9	11	23	33	11	14

TABLE II.

Staphylococci classified on colour and presumed pathogenicity.

	Gelatine.	Mannite.	Haemolysis.	Coagulase.	SEROLOGY.			Type.		
					Unstable.	Negative.	Atypical.	1	2	3
Pigmented strain *S. aureus.*										
Total 71	67	67	63	66	3	3	17	29	8	11
Percentage	94	94	89	93	4	4	24	41	11	15
Non-pigmented coagulase-positive *S. albus* pathological variants from *S. aureus.*										
Total 7	6	7	6	1	0	0	3	3	1	3
Percentage	86	100	86	100	0	0	43	43	14	43
Non-pigmented coagulase-negative *S. albus* presumably saprophytic.										
Total 23	12	3	18	..	6	8	3	1	2	2
Percentage	52	13	78	..	26	35	13	4	9	9

TABLE III.

Comparing the characteristics of the coagulase-positive (presumably pathogenic strains) with the coagulase-negative (presumably non-pathogenic).

	Pigmented.	Gelatine.	Mannite.	Hæmolysis.	SEROLOGY.					
					Unstable.	Negative.	Atypical.	Type.		
								1	2	3
Coagulase-positive presumably pathogenic.										
Total 73	66	68	70	64	3	1	19	32	8	10
Percentage	*91*	*93*	*96*	*88*	*4*	*1·3*	*26*	*44*	*11*	*14*
Coagulase-negative presumably non-pathogenic.										
Total 28	7	5	3	9	7	9	4	1	3	4
Percentage	*25*	*18*	*11*	*33*	*25*	*32*	*14*	*3·6*	*11*	*14*

TABLE IV.

Showing number of strains from different sources and their coagulase and pigment characteristics.

| | FROM PATHOLOGICAL SOURCES. TOTAL 84. | | FROM HEALTHY SKIN. TOTAL 17. | |
	Number.	Per cent.	Number.	Per cent.
Pigmented and coagulase-positive	64	*76*	2	*15*
Pigmented and coagulase-negative	6	*7*	1	*8*
Non-pigmented and coagulase-positive	6	*7*	1	*8*
Non-pigmented and coagulase-negative	8	*9·5*	13	*76*

OXIDATIONS EFFECTED BY THE PLAGUE BACILLUS.

BY

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INTRODUCTION.

IN a previous communication dealing with the metabolism of the plague bacillus the nutritional requirements of the organism were reported (Rao, 1939). In the present paper are described manometric experiments on the oxidations catalysed by suspensions of the bacillus. The main object has been to collect quantitative data on the ability of the organism to oxidize and thus utilize as energy sources the more important sugars, organic acids and amino-acids met with in the environment. This information serves also as a necessary preliminary to further investigations on aspects of bacterial oxidation and reduction. The experiments are concerned only with the question whether a given compound is oxidized and of the rate of oxidations under defined conditions. The nature of the oxidation products, the path of break-down, and other details being outside the scope are not considered. The use of washed suspensions for the study of bacterial metabolism (the so-called 'resting cell' technique) that is used here has the following important and recognized advantages over other methods: the bacterial cells are in a condition unfavourable for multiplication so that respiratory activity can be studied to a large extent in isolation from activity associated with growth: the suspension, which can be standardized, behaves like an enzyme solution: a single substrate can be studied at a time : manometric and microchemical methods can be applied to obtain quantitative results. The method is almost indispensable for the investigation of a micro-organism like the plague bacillus the metabolism of which is very little known.

TECHNIQUE.

Bacterial suspensions.—The plague strain ' 120/5H, virulent ' (which has been selected and used for studying other aspects of metabolism as well) was used for all the experiments. Some of the experiments were repeated with another strain, ' I, virulent ', of a different origin and were found to yield results similar in all

important respects. The strain was transferred from the stock blood-agar sub-culture into 200 c.c. acid-digest broth and grown for a week. The broth-culture, after testing purity, was stored in the refrigerator and was used for plating Roux bottles (5 c.c. culture per bottle) containing acid-digest broth at pH 6·9 with 2 per cent agar. After 48 hours' incubation at 27°C. the thick bacterial growth from the agar surface was dislodged and collected in 0·85 per cent saline by rolling a few spherical and smooth glass-beads. The suspension was centrifuged at 3,000 r.p.m. for 15 minutes and the sedimented bacteria were re-suspended in fresh saline. They were not washed further since certain oxidation catalysts are lost during the process (unpublished experiments). The suspension was shaken with glass-beads to break up clumps and its bacterial content was determined by drying 1·00 c.c. at 100°C. for one hour and weighing the residue accurately. The weight of the bacteria in the dry residue is obtained by deducting its content of NaCl (8·5 mg.) from the total weight. The suspension was diluted further with saline to contain 20 mg. bacteria, dry weight, per c.c. Each Roux bottle produces about 100 mg. bacteria or 5 c.c. standard suspension. The suspension was prepared fresh each day since its activity falls even in the refrigerator.

Manometric methods.—The oxidation reactions were studied with Warburg manometers which were shaken at the rate of 100 oscillations per minute in a water-bath maintained electrically at $27 \pm 0.05°C$. Conical manometric vessels of 15 c.c. to 17 c.c. capacity with side bulbs were used. Each vessel contained the following quantities of solutions: the side bulb contained 0·50 c.c. bacterial suspension containing 10 mg. bacteria; the central well contained 0·10 c.c. 20 per cent KOH with a small roll of filter-paper; the main chamber contained 0·10 c.c. distilled water, 2·00 c.c. of the substrate solution (or in the controls 2·00 c.c. distilled water) and 0·30 c.c. of M/1·5 phosphate buffer at pH 7·0; the total volume being 3·00 c.c.; the gas-space consisted of air. After 15 to 20 minutes' shaking for temperature equilibration, the bacteria were tipped into the main chamber, which marked the zero minute of the reaction. A thermo-barometer and a control manometer (with bacteria but no substrate) were always run with every batch of manometers. Further details of manometric technique were exactly as described by Dixon (1934).

Substrates.—In Table I are listed all the compounds which were tested for bacterial oxidation. They were chemically pure, natural or synthetic, samples supplied by Merck, Kahlbaum, or Schuchardt, or prepared in the laboratory. A few of them were further purified. The substrate concentration was 0·05 molar with three exceptions, which were: (1) ethyl alcohol which is inhibitory at this concentration, (2) cystine which is not soluble to the extent required at the necessary pH, and (3) cystein hydrochloride which was used at the same concentration as cystine for comparison. These three compounds were used at a uniform concentration of 4 mg. per vessel. Tyrosine which is insoluble was used as a fine suspension. All the substrates were neutralized to pH 7·0 (phenol red) with NaOH or HCl before use. In the absence of a complete series of samples of the optical isomers of the amino-acids little light could be thrown on optical specificity.

Expression of results.—Oxidation is indicated and measured by the increased oxygen uptake of the bacteria in the presence of the substrate. The O_2-uptake of the bacteria in the absence of substrate was determined in a separate control

manometer for every parallel series of manometers, and subtracted from the O_2-uptakes observed with each substrate in that series to obtain that due to the oxidation of the substrates. This is expressed as cu. mm. oxygen (at N. T. P.) taken up by 10 mg. bacteria at a substrate concentration of 0·05 M. and 27°C. and corrected for control. Oxidation curves obtained by plotting O_2-uptake in cu. mm. against time of reaction in minutes are presented for all those substrates which are oxidized at appreciable rates. (In Graph 1 are given the oxidation curves for carbohydrates, in Graph 2 for amino-acids, and in Graph 3 for organic acids.) The *rate* of oxidation has been expressed as the metabolic quotient, Q_{O_2} (*see* Table I).

$$Q_{O_2} = \frac{\text{Cu. mm. oxygen absorbed}}{\text{mg. bacteria} \times \text{hours}}$$

= cu. mm. oxygen absorbed by 1 mg. bacteria in 1 hour. From the oxidation curves it can be seen that during the one hour that the reaction has been followed the rate varies considerably from point to point. The Q_{O_2} values of Table I have therefore been calculated for the entire period and represent the average rates of oxidation for the first hour.

TABLE I.

The rates of oxidation of substrates by the plague bacillus.

Substrate.	Q_{O_2}	Substrate.	Q_{O_2}
d (+)-mannose	36·1	dl-proline	9·79
d (+)-glucose	35·8	succinate	7·30
d (−)-fructose	34·6	l-cystine (4 mg.)	5·35
d (+)-galactose	31·5	d-glutamate	5·20
dl-lactate	29·1	l (+)-arabinose	4·90
hexosediphosphate	28·5	glycine	4·88
maltose	27·4	dl-cystine (4 mg.)	3·87
pyruvate	21·6	cystein HCl (4 mg.)	3·48
d-mannitol	18·1	citrate	3·21
dl-serine	14·6	dl-phenylalanine	3·20
dl-alanine	13·0	l-tyrosine	2·87
acetate	11·0	lactose	2·73
fumarate	10·3	ethyl alcohol (4 mg.)	2·61
l-malate	9·85	d (+)-tartrate	2·52

TABLE I—*concld.*

Substrate.	Q_{O_2}	Substrate.	Q_{O_2}
l (+)-rhamnose	2·43	glycerol	0
dl-methionine	1·92	formate	0
d (+)-xylose	1·59	propionate	0
sucrose	1·56	butyrate	0
d dulcitol	1·19	capronate	0
dl-isoleucine	1·13	*dl*-aspartate	0
dl-valine	0·89	*l*-lysine	0
dl-leucine	0·16	*l*-histidine	0
raffinose	0	*d*-arginine	0
d-sorbitol	0	*l*-tryptophane	0

RESULTS AND DISCUSSION.

In Table I is given the list of compounds tested for oxidation by the bacillus arranged in the decreasing order of their Q_{O_2} values. In the discussion that follows the chemical structure of the substrate compounds is given so as to convey, as far as possible, the relation of structure to oxidizability. Towards 12 out of the 48 compounds tested the bacillus possesses no oxidative activity whatever. Several others are oxidized only to a slight extent. The highest activity recorded is displayed towards the hexose sugars (*see* Graph 1).

Glucose, fructose, and mannose, are oxidized at approximately equal rates, and galactose at a slightly lower rate. The disaccharide maltose (or glucose-4-*a*-glucoside) is oxidized vigorously, lactose (or glucose-4-*β*-galactoside) moderately, and sucrose (or 1-*a*-glucoside-2-*β*-fructofuranose) slightly. The triasaccharide raffinose (or 2-*β*-fructofuranose-1-*a*-glucose-6-*a*-galactoside) is not oxidized.* Oxidative activity towards the pentoses is considerably less than that towards the hexoses. Arabinose is oxidized moderately; the methyl-pentose, rhamnose, at half this rate, and xylose at a still lower rate. Of the sugar-alcohols mannitol is oxidized rapidly, but dulcitol is oxidized slightly, while sorbitol is not attacked. Hexosediphosphate (1 : 6-diphosphofructofuranose) is oxidized at a rate slightly below that of the hexoses. This is a fact of considerable interest in view of the recent demonstration that in the biological break-down of sugar through phosporylated intermediates hexosediphosphate holds a central position.

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*A rapid oxidation observed with a sample of raffinose was found to be due to the presence of a trace of glucose as an impurity. The sugar when freed from this impurity was not oxidized.

GRAPH 1.
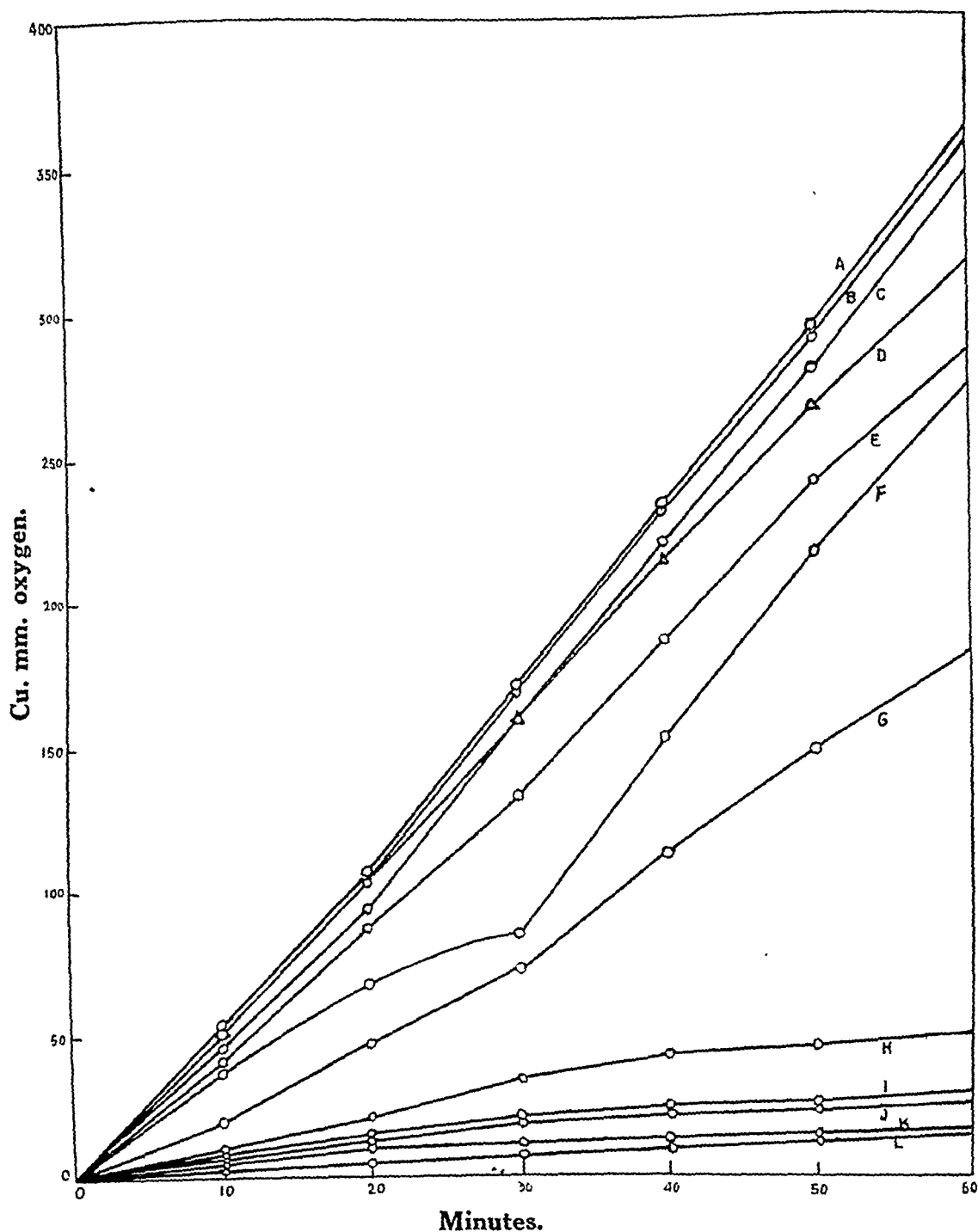
OXIDATION OF CARBOHYDRATES.—A-mannose; B-glucose; C-fructose; D-galactose; E-hexosediphosphate; F-maltose; G-mannitol; H-arabinose; I-lactose; J-rhamnose; K-xylose; and L-sucrose.

The amino-acids are oxidized by the plague bacillus at rates which are in general lower than those of carbohydrates (*see* Graph 2). Glycine [$CH_2(NH_2).COOH$] is oxidized at a moderate rate. Alanine [$CH_3.CH(NH_2).COOH$] is attacked rapidly. Aspartic acid [$HOOC.CH_2.CH(NH_2).COOH$] is not oxidized but glutamic acid [$HOOC.CH_2.CH_2.CH(NH_2).COOH$] is oxidized. Serine [$CH_2OH.CH(NH_2).COOH$] has the highest oxidation rate among the amino-acids. Cystine [$HOOC.CH(NH_2).CH_2S—SH_2C.CH(NH_2).COOH$] and cystein [$CH_2SH.CH(NH_2).COOH$] are both oxidized. *l*-cystine is oxidized at a higher rate than *dl*-cystine indicating that the natural isomer (*l*-) is preferentially attacked. Methionine [$CH_3.S.CH_2.CH_2.CH(NH_2).COOH$] is attacked at a slow rate. The amino-acids with aromatic nuclei, phenylalanine [$C_6H_5.CH_2.CH(NH_2).COOH$], and tyrosine [$HO.C_6H_4.CH_2.CH(NH_2).COOH$], are both oxidized at nearly equal rates though the latter is almost insoluble at pH 7. Proline (α-pyrrolidine carboxylic acid) is oxidized rapidly. Tryptophane (β-indole-α-aminopropionic acid) is, however, not attacked. It is of interest to compare this with the known observation that the indole test is always negative with the plague bacillus. Isoleucine and valine are slightly and leucine is hardly oxidized. Lysine, histidine, and arginine, are left untouched. It should be noted that the amino-acids essential for growth, viz., proline, phenylalanine, and cystine (Rao, *loc. cit.*), are all oxidized by the bacillus.

The biologically important organic acids tested are oxidized at rates which are in general higher than those of amino-acids but lower than those of sugars (*see* Graph 3). Lactic acid [$CH_3.CHOH.COOH$] has next to the hexoses the highest oxidation rate. Pyruvic acid [$CH_3.CO.COOH$] is also oxidized at a high rate. Though acetic acid [$CH_3.COOH$] is oxidized at a good rate none of the other monobasic saturated acids tested, formic, propionic, butyric, and capronic, are attacked. Fumaric acid [$HOOC.CH=HC.COOH$], malic acid [$HOOC.CH_2.CHOH.COOH$], and succinic acid [$HOOC.CH_2.CH_2.COOH$], are oxidized at nearly equal rates. But citric acid [$HOOC.CH_2.C.OH.COOH.CH_2.COOH$] and tartaric acid [$HOOC.CHOH.CHOH.COOH$] are oxidized to a slight extent only.

Of the remaining compounds glycerol [$CH_2OH.CHOH.CH_2OH$] is not oxidized. Ethyl alcohol [$CH_3.CH_2OH$] is inhibitory at 0·05 M. concentration but is oxidized at lower concentrations.

It is pertinent to inquire how far the oxidations studied are paralleled by the fermentation reactions described in the bacteriological literature. Oxidation in which molecular oxygen is the final hydrogen-acceptor is different from fermentation or break-down in the absence of oxygen, particularly with reference to the end-products. However, a basis of comparison is possible since the fundamental condition for the occurrence of either reaction is the *activation* of the substrate. When an organism ferments a substrate it should be expected to oxidize it as well and vice versa, though the production of acidity which accompanies the fermentation of carbohydrates and some other compounds, and which is taken as the main evidence for the occurrence of fermentation in bacteriological tests, may not take place with several other types of substrates. Petrie (1929) has summarized in a tabular

GRAPH 2.
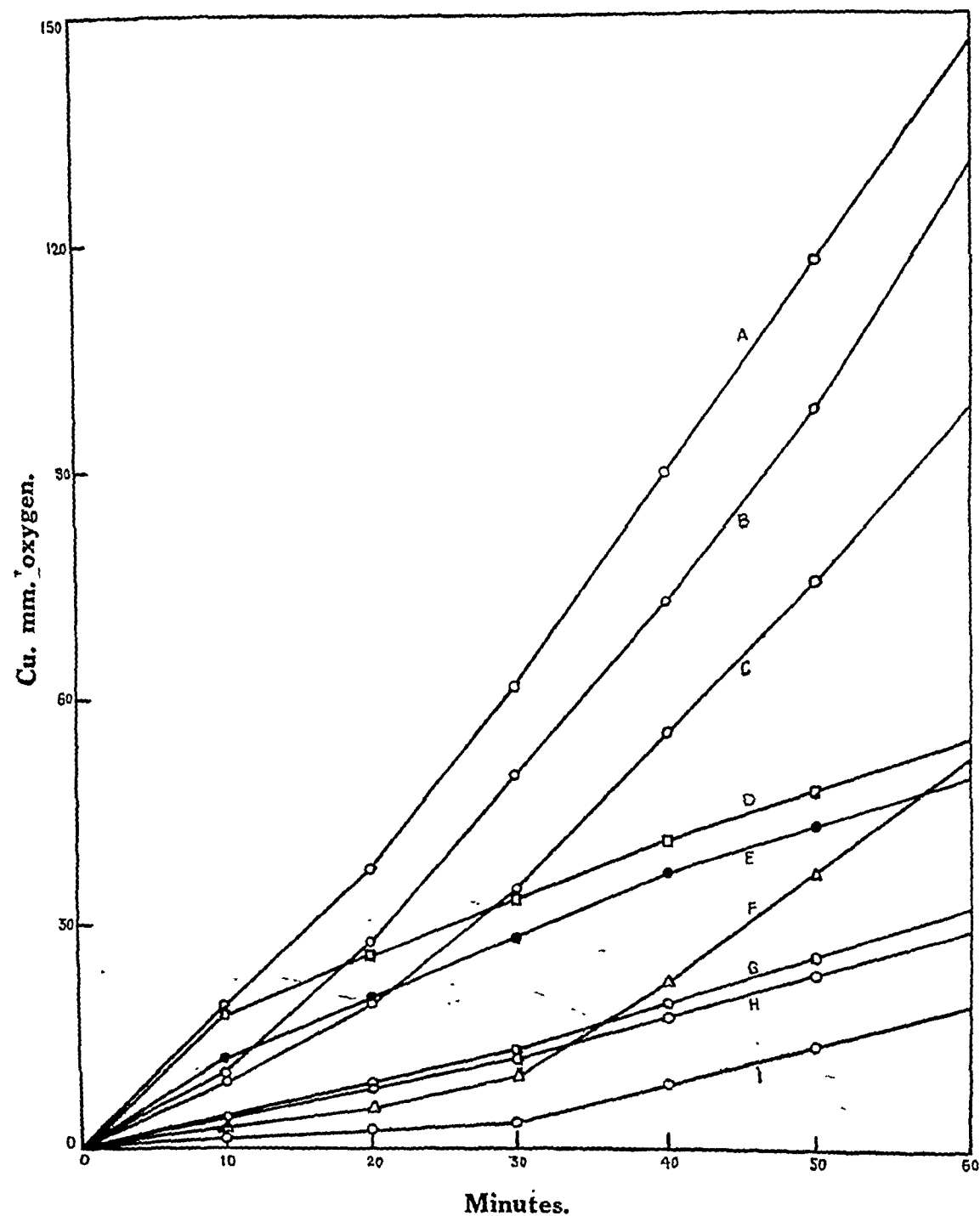
OXIDATION OF AMINO-ACIDS.—A-serine ; B-alanine ; C-proline ; D-*l*-cystine ; E-glycine ; F-glutamic acid ; G-phenylalanine ; H-tyrosine ; and I-methionine.

GRAPH 3.
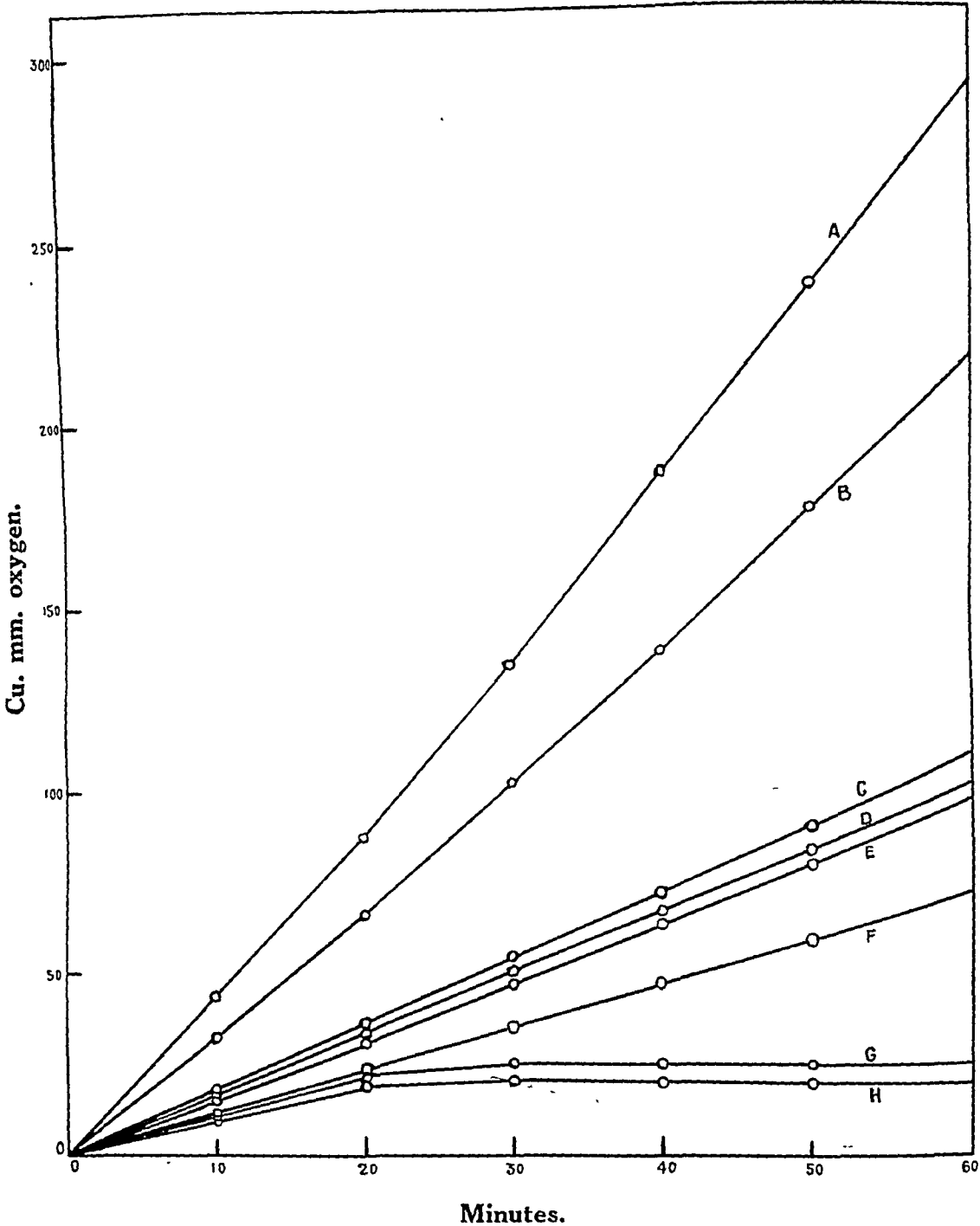
OXIDATION OF ORGANIC ACIDS.—A-lactic acid ; B-pyruvic acid ; C-acetic acid ; D-fumaric acid ;
E-malic acid ; F-succinic acid ; G-citric acid ; and H-tartaric acid.

form 12 sets of observations by different workers on the fermentation reactions (acid production) of the plague bacillus. The observations relating to such of the compounds studied here have been re-cast in Table II and presented side by side with the oxidation rates :—

TABLE II.

A comparison of oxidation with fermentation reactions of the plague bacillus.

Observations on fermentation adapted from Petrie (1929).

Substrate.	OXIDATION.	FERMENTATION.	
	Q_{O_2}	Number of times acid production observed.	Total number of observations.
Glucose ..	35·8	12	12
Fructose ..	34·6	11	11
Galactose ..	31·5	10	11
Maltose ..	27·4	10	12
Mannitol ..	18·1	11	12
Arabinose ..	4·90	3	5
Lactose ..	2·73	0	12
Rhamnose ..	2·43	1	1
Xylose ..	1·59	1	2
Sucrose ..	1·56	0	12
Dulcitol ..	1·19	1	9
Raffinose ..	0	0	6
Sorbitol ..	0	0	4
Glycerol ..	0	3	6

A strict comparison of the two types of data is difficult on account of the fact that many of the observations on fermentation are not consistent, due either to real differences in the activities of the different strains or apparent differences caused by wide variations in the technique, and of the fact that the observations are purely qualitative. A general parallel between oxidative and fermentative abilities of the plague bacillus is evident. Manometric experiments clearly show a definite measurable action on substrates like lactose and sucrose though fermentation tests reveal no action whatever.

The study of oxidation conclusively indicates which of the several compounds occurring in the culture media are available to the plague bacillus as energy sources and which of them are metabolized. Glucose or lactic acid are the best (and the least expensive) carbon sources that can be used to supplement media. Of the amino-acids the following only are of service as nitrogen and energy sources, and consequently only these can be added to enrich media: serine, alanine, proline, cystine, glutamic acid, glycine, phenylalanine, tyrosine, and methionine. The two synthetic protein-free media described by the author previously (Rao, *loc. cit.*) can be modified in the light of these facts.

SUMMARY.

1. The oxidation of forty-eight compounds (carbohydrates, amino-acids, and organic acids) by suspensions of strain ' 120/5H, virulent' of the plague bacillus has been studied manometrically in air at 27°C. and 0·05 M. substrate concentration. The metabolic quotient, Q_{O_2}, has been calculated in each case and oxidation curves have been constructed.

2. The oxidative activity of the bacillus is greater towards carbohydrates (Q_{O_2} 36·1 to 1·59) than towards organic acids (Q_{O_2} 29·1 to 2·52) or towards the amino-acids (Q_{O_2} 14·6 to 0·16). Only thirty-six of the compounds tested are oxidized.

3. Substrates which undergo pronounced oxidation fall in the following series of relative oxidation rates :—

Carbohydrates : mannose > glucose > fructose > galactose > hexosediphosphate > maltose > mannitol > arabinose > lactose > rhamnose > xylose > sucrose > dulcitol.

Amino-acids : serine > alanine > proline > cystine > glutamate > glycine > phenylalanine > tyrosine > methionine.

Organic acids : lactate > pyruvate > acetate > fumarate > malate > succinate > citrate > tartrate.

4. In the light of these experiments it is concluded that the best energy and carbon sources to supplement media are glucose and lactate. The amino-acids which are available to the bacillus as nitrogen sources are: serine, alanine, proline, cystine, glutamic acid, glycine, phenylalanine, tyrosine, and methionine.

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ACTION OF METHYLENE BLUE ON *MYCOBACTERIUM LEPRÆ MURIS.*

BY

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THE introduction of the methylene-blue treatment for leprosy by Montel (1934) directed attention to the nature of the action of the dye on *Myco. lepræ*. Dorolle and Ngo-Quang-Ly (1935), while reporting improvement in the clinical symptoms, found that the dye treatment had very little action on the lepra bacilli; only in two out of the 18 cases did the bacilli disappear from the nose and in no case from the cutaneous lesions. Lèpine and Markianos (1935) studied the action of methylene blue on the morphology and the staining properties of the bacillus. They punctured the leprous lesions after intravenous injections of the dye and stained the exudate by Ziehl-Neelsen method. The bacilli stained blue and not red and some of them looked granular. Lèpine and Markianos attributed these changes to the direct degenerating action of the dye on the bacilli.

Due to obvious difficulties in investigating the action of the dye *in vitro* on *Myco. lepræ*, attention was diverted to the allied organism, *Myco. lepræ muris*— the causative organism of rat leprosy. Berny (1935) prepared an emulsion of rat leproma from an infected rat and treated it with 1 per cent solution of methylene blue for 12 to 24 hours. The treated emulsion was injected into six rats, only two of which survived the dose. These two developed rat leprosy in due course. The dye, therefore, had no attenuating effect on the organism. Marchoux and Chorine (1935) investigated the *in vivo* action of the dye on the rat-leprosy bacillus. Experimentally infected rats were injected intravenously with the dye solution and were sacrificed after varying intervals for microscopic examination. They found that, while in the living cells the stain is reduced to a colourless product, the rat-leprosy bacilli retain the blue colour. They suggested that the dye, although in itself not lethal to the organism, may serve to convey an active substance into them

THE PRESENT WORK.

The following experiments were undertaken with a view to verifying the findings regarding the *in vitro* and *in vivo* action of methylene blue on the rat-leprosy bacillus :—

The in vitro *action of methylene blue on rat-leprosy bacillus.*

An emulsion rich in rat-leprosy bacillus was prepared from the liver of an experimentally infected rat. The emulsion was divided into three parts, to one part was added equal amount of 1 per cent aqueous solution of methylene blue, to the second saturated solution (2 per cent) of the dye was added in the proportion of five drops to 1 c.c. of the emulsion, and the third part was not treated in any way and served as a control. The dye solutions were sterilized in an autoclave, as in a preliminary experiment in which unsterilized dye solution was used most of the animals died. Before being injected into rats the treated and the untreated emulsions were kept at 37°C. for two hours. Ten rats were inoculated with each of the treated emulsions and twenty with the untreated emulsion. Each rat received 1 c.c. of the emulsion, 0·5 c.c. subcutaneously into the right groin and 0·5 c.c. intra-peritoneally. When any animal died a post-mortem examination was done and smears taken from the different organs were stained with Ziehl-Neelsen stain to ascertain whether or not there was generalized infection. The rats that died before the expiry of three months were not taken into account as the disease rarely develops before that period.

Results.— Out of the twenty rats inoculated with untreated emulsion 14 survived for three months and longer. Twelve out of the fourteen were examined and showed generalized infection. Of the 10 rats injected with the emulsion treated with equal amounts of 1 per cent methylene-blue solution, six survived beyond three months. Four of them showed generalized infection and two only localized infection; these two had lived only a little beyond three months. Of the 10 animals injected with the emulsion treated with saturated solution of the dye, six survived beyond three months and all of them showed generalized infection. These results are shown in the Table :—

TABLE.

Nature of emulsion.	Number of rats inoculated.	Number survived beyond three months.	Number showing generalized infection.	REMARKS.
Emulsion treated with equal part of 1 per cent solution of methylene blue.	10	6	4	Two lived only a little over three months.
Emulsion treated with 2 per cent solution of methylene blue (5 drops to 1 c.c. of emulsion).	10	6	6	..
Untreated emulsion ..	20	14	12	Two rats were not examined.

The experiment showed that the dye had no attenuating effect on the rat-leprosy bacillus.

The action of the dye on the staining properties of the bacillus *in vitro* was next studied. An emulsion of the organism was mixed with an equal part of 1 per cent solution of methylene blue. The mixture was kept in the incubator at 37°C. and smears were taken every few hours up to 48 hours. These smears were stained by the Ziehl-Neelsen method. The bacilli were stained red as usual and not blue.

The in vivo *action of methylene blue on* Myco. lepræ muris.

A sterile 1 per cent solution of methylene blue was injected intraperitoneally into 10 rats experimentally infected with rat leprosy about six months previously. Four of the rats received a single injection of 1 c.c. each and were sacrificed at the end of 1, 3, and 6 hours respectively, two being sacrificed at the end of 6 hours. Another four received a single injection of 0·5 c.c. and were sacrificed at the end of 1, 2, 3, and 7 days. One rat received three injections of 0·5 c.c. within two weeks and was sacrificed at the end of that period. The remaining one received five injections of 0·5 c.c. within three weeks and was sacrificed at the end of that period.

Examination.—When a rat was sacrificed three smears were made from each of the following organs : viz., liver, spleen, omentum, inguinal, axillary, and cervical glands. One smear was examined unstained to detect any blue bacilli, the other was stained with Ziehl-Neelsen method but, after decolorization, the counter-staining with methylene blue was omitted, and the third smear was stained by the usual Ziehl-Neelsen method. In addition to making smears, frozen and paraffin sections from the different organs were made in a few instances.

Results.—No change in the morphology of the bacillus was found in any of the smears or sections.

In unstained smears and in frozen sections examined unstained, no blue bacilli were encountered.

In sections stained by the Ziehl-Neelsen method, the bacilli were always red.

In smears stained with carbol-fuchsin and decolorized with acid-alcohol, the bacilli were always bright red.

In smears stained as above but counterstained with methylene blue after decolorization, i.e., according to the usual Ziehl-Neelsen method, the bacilli were usually red. In some smears, however, the red colour of the bacilli was not so bright and was to some extent masked by the blue colour. This appearance was most marked in smears from a rat killed six hours after the methylene-blue injection. The experiment was repeated. Another rat was injected with 1 c.c. of the dye solution and sacrificed six hours later. No masking of the red colour of the bacilli in smears from this rat stained by the Ziehl-Neelsen method was seen. The masking of the red colour in the previous case appears to have been due to overstaining with methylene blue, as in smears that were not counterstained with methylene blue after decolorization the bacilli looked quite red.

CONCLUSIONS.

1. *In vitro* methylene blue has no attenuating effect on *Myco. lepræ muris.* Berny's findings on this point have been confirmed.

2. *In vitro* methylene blue has no action on the acid-fast property of *Myco. lepræ muris.*

3. *In vivo* methylene blue has no degenerating action on *Myco. lepræ muris* and does not in any way change its morphology or staining properties. Even repeated injections of the dye have no effect in this respect. We have not been able to confirm the findings of Marchoux and Chorine in this connection.

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A STUDY OF THE LESIONS IN RHEUMATIC HEART DISEASE IN SOUTH INDIA.

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INTRODUCTION.

IT has been asserted (Clarke, 1930) that rheumatic infection does not occur in the Tropics, i.e., in the area which lies between 23° and 28″ North and South of the Equator. This statement has gained wide currency in the medical literature on diseases in the Tropics and in textbooks of medicine. But a review of the subsequent Indian medical literature on the subject disproves this assertion. Hughes and Yusuf (1930) reported 24 cases of cardiac rheumatism from Lahore. They came to the conclusion that, in the Punjab rheumatism is an important cause of valvular heart disease, especially mitral stenosis. Stott (1930) from Lucknow reported 20 cases of chronic heart failure in young Indian males, mostly between 12 and 18 years of age following progressive stenosis of the mitral valve. Hodges (1932) from Jalpaiguri, Bengal, reported eight cases under 12 years of age showing the rarer features of rheumatic infection. He stated there seems to be ample evidence, that, as far as India is concerned, rheumatic infection is by no means rare and is a potent factor in the production of heart disease. Banerjea (1935) reported a further series of 25 cases of rheumatic heart disease in childhood from Bengal. Mangalik and Stott (1938) and Stott (1938) published critical studies on the incidence of rheumatic infection in India, particularly from Agra and Lucknow. Clarke has objected to the above evidence, as the Punjab, the United Provinces, and Bengal, are not situated in the true Tropics, but to the north of this limit. South India is wholly within the Tropics, and any evidence from this area as to the incidence of rheumatic infection would be of great weight. Kamath (1931) stated that if the presence of mitral stenosis is to be taken as an index to the existence of rheumatic infection then the condition is common in South India. The present writer in 1935 published a critical study of the incidence of rheumatic infection in the Vizagapatam district in South India, with a report of 50 cases of juvenile rheumatism (Kutumbiah, 1935). In this study he adduced proof as to the common occurrence of rheumatic infection in the Vizagapatam district in South India and showed that the contention that there is no rheumatic infection in the Tropics was no longer tenable.

The literature on rheumatic infection in India has hitherto been mainly clinical. Records of autopsies on subjects of rheumatic infection are very rare in Indian medical literature. Rogers (1930) stated that of 4,800 post-mortem examinations in Calcutta only one case of rheumatic carditis was found in 37 years. This rarity of autopsies has been adduced by Clarke (*loc. cit.*) as proof of his contention that there is no rheumatic infection in the Tropics. He writes, ' it can hardly be accepted that rheumatic fever occurs in a country where mitral stenosis is never found in the post-mortem room. The absence or presence of mitral stenosis has a' big bearing on this question of the presence or absence of rheumatic fever. Proof that the disease does arise in the Tropics can easily be given by sending some hearts with mitral stenosis. But they must be from people who were born in the Tropics and have never been outside'. This rarity of autopsy records is surprising as death from chronic rheumatic heart disease is by no means uncommon in our hospitals. Statistics as to the mortality rate of rheumatic infection in India is not available in literature, but statistics as to the number of post mortems on subjects of rheumatic infection is available for the hospitals of Agra and Lucknow. Mangalik and Stott (*loc. cit.*) have recorded 6 (1930-1936) and 15 (1914-1937) cases respectively. All these cases revealed lesions of the mitral valves—mostly mitral stenosis. The mortality rate of rheumatic infection in Vizagapatam for the period of 1935-1938 based on the records of the King George Hospital is 8·8 per cent. The number of cases of rheumatic infection admitted into the wards during 1935-1938 was 284. The number of deaths during this period from the above cause was 25. Of this number autopsy was possible on 10 and the youngest subject in this series was aged 18 years. It is evident from the above statistics that autopsies on subjects of rheumatic infection are not rare in Vizagapatam. The rarity in other provinces must be due not to the rarity of rheumatic infection, but to the well-known difficulty of obtaining permission to do autopsies particularly on children. While autopsy records are rare histopathological studies are unknown on this subject in Indian medical literature. Muthayya has, at the 14th All-India Medical Conference held at Madras in 1937, exhibited slides showing myocardial Aschoff nodules from a Hindu boy who died of rheumatic carditis. This has not, however, attracted the attention it merited.

Hitherto in India reliance has been placed on such gross pathological lesions as mitral stenosis and vegetations as the most reliable evidences of rheumatic infection. How far are we to regard mitral stenosis as evidence of rheumatic infection ? Coombes (1924) asserted that every case of mitral disease must be regarded as a case of advanced cardiac rheumatism. Stott (1930) reviewed critically the possible causes of mitral stenosis and concluded that unrecognized subacute rheumatic infection in childhood was the probable cause of mitral stenosis in young Indian adults. But histopathology alone can supply the most reliable evidence for this. The researches of Aschoff (1904) have led to the recognition of the Aschoff bodies as the specific lesion of rheumatic disease. They are not invariably present in rheumatic infection, especially during the less active stages. The recent studies of Gross and Friedberg (1936) have made it possible to define rheumatic from non-rheumatic hearts, even in the absence of Aschoff's bodies or a typical clinical history. In our autopsy room it is rare to come across active cases of rheumatic infection, but most of our cases are active cases where death has been caused by decompensation without clinical evidence of a final acute attack often with no previous

history of rheumatic fever; others are inactive cases of chronic valvular disease of the typical rheumatic variety. In the recognition of these cases, histopathological study is absolutely essential.

This article presents the histopathological evidence derived from autopsies conducted in South India on rheumatic subjects to prove conclusively the incidence of rheumatic infection in South India and so in the Tropics.

MATERIAL AND METHODS.

The material for this study consisted of six rheumatic hearts out of the 14 available ones. Five of these were obtained from post mortems conducted on cases which were under observation and treatment in the medical wards of the King George Hospital, Vizagapatam. Of these two were from Hindu males and three from Hindu females, all residents of the Vizagapatam district who have never been outside this district. The sections of the sixth specimen were obtained through the courtesy of Dr. Muthayya of the Madras Medical College. It was also from a Hindu boy aged ten years resident in Madras and who died in the General Hospital, Madras, from rheumatic carditis. Out of these six specimens two had to be discarded, though they showed advanced mitral stenosis, as there was evidence of co-existing syphilitic infection. Based on the criteria of activity and inactivity outlined by Rothschild, Kugel and Gross (1933), Gross and Friedberg (*loc. cit.*) after a careful study of the clinical records and pathological specimens, divide cases of rheumatic heart disease into six groups. The above four cases fall into the following of their groups :—

Active cases where one preceding attack occurred within one year of the fatal outcome—two cases (group II of Gross and Friedberg).

Active cases where death was caused by decompensation without clinical evidence of a final acute attack—two cases. In these cases there was no previous history of rheumatic fever (group V of Gross and Friedberg).

The sections for this study were cut according to the standardized technique of Gross, Antopol and Sacks (1930). According to this technique six blocks are cut which show a maximum number of lesions in a minimum number of blocks of tissue. These blocks are cut from definite topographic sites in the heart and are so designed that each block includes certain 'strategic' sites, i.e., areas which experience has shown are frequently the seat of disease. The heart is opened in the customary manner and after fixation the following blocks are cut :—

(1) Left auricle (L.A.); (2) mitral posterior (M.P.); (3) posterior papillary muscle, left (P.P.M.); (4) aorta, aortic valve and mitral valve (A.M.V.); (5) pulmonary artery and valve (P.A.V.); and (6) tricuspid valve and septum (T.V.).

The following staining methods have been used in the present investigation :—

(1) Ehrlich's acid-hæmotoxylin and eosin—for routine examination.

(2) Weigart's iron-hæmotoxylin and van Gieson's stain—for staining connective tissue.

(3) Orcin stain—for staining elastic tissue.

(4) Masson's trichrome stain—for the demonstration of collagen.

(5) Heidenhain-Mallory-Azan stain—for the demonstration of collagen.

(6) Wilder's modification of Foot-Bielschowsky stain—for the demonstration of reticulum.

Morbid Anatomy and Histopathology.

Results of the study.

Case I. H. M. C., 10 years.

Morbid anatomy.—The heart with pericardium weighed 16 oz. It appeared to be very much enlarged *in situ*. The parietal layer of the pericardium was bound down to the visceral layer by adhesions, the pericardial space being completely obliterated. The adhesions were broken down without much difficulty. On the inner surface of the parietal layer and on the surface of the heart were seen numerous whitish grey nodules varying in size from a pin-head to a pea. In some places a number of these nodules had coalesced together. The right ventricle was enlarged. The tricuspid orifice admitted two fingers. A few, firm, tiny, warty vegetations were present on the ventricular aspect of the cusps of the aortic valve. The mitral cusps were thickened along the lines of contact and the orifice admitted the tips of two fingers. The papillary muscles of the ventricle showed well-marked 'tabby cat' appearance.

Histopathology.—*Section I.* (L. A.) Pericardium thickened. On its surface there is a fibrinous exudate which is being organized. Beneath is vascular granulation tissue. Some of the vessels show intimal thickening and sub-intimal proliferation with marked narrowing of the lumen. Here and there, there are collections of cells—mostly lymphocytes and a few plasma cells (Plate XXV, fig. 1). The endocardium is markedly thickened. The deeper layers of endocardium show sparse chronic inflammatory infiltration. The auricular myocardium shows patches of hyaline degeneration and the muscle-fibres are separated by an œdematous infiltration. Myocardium shows a sparse lymphocytic infiltration mostly perivascular in distribution.

Section II. (M. P.) The auricular endocardium is markedly thickened and shows hyaline degeneration. The deeper layers show marked vascularization—mostly capillaries and infiltration with inflammatory cells—lymphocytes—particularly round the blood vessels in the sub-endocardial connective tissue. *Valve angle.* Tongue-like projection into the angle due to reduplication of the endothelium. The surface endothelium shows evidence of proliferation. *Valve ring* vascularized. A good proportion of the vessels have musculo-elastic walls. Some even show endarteritic changes—marked perivascular infiltration with lymphatic cells round some vessels. Spongiosum of the valves markedly thickened (Plate XXV, fig. 3). *Myocardium* (ventricular): Numerous Aschoff bodies in various stages of evolution are seen. Some of these are para-arterial, while some are situated in the muscle (Plate XXVI, figs 5 and 6, and Plate XXVII, fig. 8).

Section III. (P. P. M.) Shows a typical Aschoff nodule situated para-arterially (Plate XXVII, fig 9).

Section IV. (A. M. V.) *Aortic valve :* Fibrosa thickened. The valve angle shows a tongue-like projection. Ring spongiosa shows vascularization—vessels mostly thin-walled except one or two which show muscular fibres in the walls. There is a fairly conspicuous infiltration in the spongiosa with cells—lymphocytes, few fibroblasts and histiocytes—chiefly perivascular (Plate XXV, fig. 2). Ring annulus shows marked vascularization—vessels thick-walled and of musculo-elastic variety with narrowing of the lumen—infiltration less marked than in ring spongiosa—perivascular (Plate XXVI, fig. 4). Cells mostly lymphocytes, few polymorphs and histiocytes. Intervalvular fibrosa shows no infiltration but a few thin-walled capillaries seen. *Left auricular endocardium :* Thickened, sub-endocardial layers show marked infiltration with lymphocytic cells and also marked vascularization—mostly thin walled capillaries. *Auricular myocardium.* Shows perivascular œdema and sparse lymphocytic infiltration.

Section V. (P. A. V.) *Pulmonary valve.* Ring spongiosa shows vascularization thin-walled with perivascular infiltration—mostly lymphocytic (Plate XXVII, fig. 7).

Section VI. (T. V.) *Tricuspid valve :* Spongiosa thickened. Valve ring shows vascularization with numerous thin-walled capillaries with moderate perivascular infiltration—mostly lymphocytes. *Myocardium.* Shows patchy areas where the muscle fibres are replaced by fibrillar connective tissue, fibrocytes and a few histiocytes which do not stain properly—fibrosing Aschoff nodules. Some of the coronary vessels show narrowing of lumen with intimal proliferation and perivascular fibrosis.

Case II.   H. M. 18 years.

Morbid anatomy.—Heart weighed 22 oz. Pericardium showed peticheal hæmorrhages. Right ventricle hypertrophied and dilated. The right auricle dilated and the wall thin. The epicardium over the right auricle showed a fine granular deposit of organized lymph. The endocardium thickened. *The tricuspid valves :* Thickened uniformly and opaque. The auricular surface of the valves uneven and puckered. A row of minute yellowish granulous vegetations seen on the auricular surface on all the three cusps about 1 mm. from the free margin. Chordæ tendineæ free ; few vegetations on the chordæ tendineæ and tips of the papillary muscles (Plate XXVIII, fig. 11). The left ventricle is slightly dilated. The left auricle showed dilatation and marked sub-endocardial fibrosis. A stalked ball thrombus about ¾″ in diameter was lying loose in the left auricle ; another thrombus was seen in the left auricular appendix. Myocardium firm and presented a steamed appearance. *Mitral valves :* Markedly thickened throughout, adherent to each other, producing a funnel-shaped stenosis with a button-hole aperture. The auricular surface of the valves rugged. The free margins show a few granular vegetations. Chordæ tendineæ fused to the ventricular surface of the cusps. Tips of the papillary muscles are approximated close to the free margins of the slit-like opening (Plate XXVIII, fig. 12). *Pulmonary valves :* Slender, translucent ; no vegetations. *Aortic valves :* Slight thickening of the free margins ; slightly opaque.

Histopathology.—I. (L. A.) Pericardium over the left auricle normal in extent and appearance. Few pericapillary collections of lymphocytes. The individual myocardial fibres hypertrophied. There is œdematous infiltration between the fibres. Endocardium is thickened ; sub-endocardial vascularization—vessels mostly thin-walled capillaries. Areas of sub-endocardial infiltration with lymphocytes and fibroblasts.

II. (M. P.) The auricular endocardium is thickened. Sub-endocardial vascularization—vessels mostly thin-walled capillaries. Sparse sub-endocardial infiltration with lymphocytes and fibroblasts. *Valve angle :* A tongue-like projection of the endothelium is seen. *Valve ring :* Vascularized ; vessels mostly thin-walled capillaries ; and a few with musculo-elastic walls. The posterior mitral valve markedly thickened. Spongiosa very prominent. Highly vascularized. Some of the vessels show endothelial and sub-endothelial proliferation leading to occlusion of the vessels. Along the vascularized core of the valve there is a ridge of cellular infiltration. The cells are pleomorphic and vary in size. There are giant hystiocytic cells, some with multiple nuclei ; fibroblasts, histiocytes, few plasma cells and round cells are also seen (Plate XXIX, figs. 14 and 15). The interstitial connective tissue is loose and tends to show hyaline swelling suggestive of an œdematous infiltration.

V. (P. A. V.) Valve ring shows scattered vascularized areas resembling granulation tissue with pronounced perivascular infiltration with round cells and plasma cells and histiocytes.

VI. (T. V.) *Valve angle :* The endothelium shows hyaline degeneration. *Valve ring :* Vascularized ; shows numerous capillaries surrounded by lymphocytes and a few fibroblasts. *Myocardium :* Throughout the myocardium there are scattered areas of collection of histiocytes, fibrocytes polymorphs and lymphocytes without any orderly arrangement. Some of the collections show perivascular distribution, fibrillar Aschoff bodies (Plate XXIX, fig. 13).

Case III.   H. M. 33 years.

Morbid anatomy.—Heart weighed 20 oz. Pericardium distended with about 10 oz. of fluid. Heart dilated mostly to the left ; right auricular epicardium shows fine, white, granular deposits. Right ventricle—hypertrophied and dilated. The tricuspid valves : Thickened, uniformly opaque ; the free margins of the anterior cusp straight and markedly thickened. Auricular surface uneven. No vegetations. Left auricle markedly dilated : Auricular endocardium white and opaque. Mitral valves : The auricular surface opaque, markedly thickened, very uneven, rough and rugged. Well-marked ledging of the proximal ends. The valves are fused into a button-hole opening. The chordiæ tendineæ are adherent to the anterior cusp on the ventricular surface. The tips of the papillary muscles closely approximated to the slit-like opening. No vegetations. Pulmonary valves : Normal. Aortic valves : Normal.

Histopathology.—*Section I.* (L. A.). Endocardium markedly thickened and shows hyaline fibrosis. The deeper layers of endocardium show sparse chronic inflammatory infiltration, especially around the vessels. Fibrous strands extend from this layer along the vessel into the myocardium separating bundles of hypertrophic myocardial fibres, which are further loosened out by an œdematous infiltration.

Section II. (M. P.) Endocardium shows the same changes as above. Myocardium (left ventricle) : The branches of the coronary artery are dilated, tortuous, and show thickening of the periarterial connective tissue with occasional fibrositic cell collection in the same ; myocardial fibres show well-marked hypertrophic changes. The coronary vessels in the pericardium show moderate

periarterial fibrous thickening ; in places, sparse round cell infiltration is seen along the small vessels entering the myocardium from the deeper layers of the pericardium (Plate XXVIII, fig. 10). *Valve angle :* Shows commencing reduplication of the endothelium of the valve. *Valve ring :* Shows marked scarring ; moderate vascularization with thin-walled capillaries. There is a very sparse infiltration with fibroblasts.

Section VI. (T. V.) *Valve angle :* Small reduplication of the endothelium of the valve. *Valve ring :* Vascularized. Vessels mostly thin-walled capillaries ; some of them markedly dilated ; fairly heavy infiltration, mostly perivascular, seen ; cells lymphocytes. The auricular surface of the valve is wavy and in some places is thrown into villi-like projections, probably due to the fibrosis underneath. There is a loose fibrous thickening of the spongiosa. *Myocardium :* The coronary branches of the myocardium show a loose periarterial fibrosis with occasional fibrocytic and small round cell collections as described in the ventricular wall (left). In some places this fibrous tissue has invaded the myocardium converting it into an ill-defined and poorly-staining mass.

Case IV. H. F. 20 years.

Morbid anatomy.—Heart weighed 12 oz. Right auricle much dilated. Right ventricle—normal in size. Tricuspid orifice shows stenosis due to fibrosis and adhesion of the valve margins—admits tips of 2 fingers. *Tricuspid valves :* Thickened, opaque, auricular surface rough and rugged—more marked in the anterior cusp. Small granular vegetations are seen on the valve margins. Chordæ tendineæ free, slightly thickened. Auricular surface smooth with slight degree of opacity all round the orifice. Left ventricle normal in size. Left auricle hypertrophied and dilated. *Mitral valves :* Auricular surface opaque, slightly thickened, uneven, rough. Well-marked ledging of the proximal ends of the valves. The distal ends of the valves fused into a button-hole opening. *Ventricular surface :* The chordæ tendineæ shortened, thickened, and in some places adherent to the cusps. The tips of the papillary muscles closely approximated to the margins of the cusps. *Pulmonary valves :* Normal. Aortic valves : Normal.

Histopathology.—*Section I.* (L. A.) Pericardium shows a slight proliferation of endothelial cells with round cell infiltration. Marked collection of sub-pericardial fat with numerous dilated capillaries. *Myocardium :* Shows œdematous infiltration. *Endocardium :* Thickened and shows hyaline degeneration.

Section II. (M. P.) Valve distorted. Auricular endocardium markedly thickened ; deeper layers show hyaline degeneration. As it is prolonged over the valve the endocardium shows wavy bundles of eosin-staining collagen fibres (Plate XXX, fig. 16). *Valve ring :* Vascularized—vessel walls of the musculo-elastic type with obliteration of the lumen. A small collection of cells mostly lymphocytes. The spongiosa of the valve is converted into a hyaline mass without any definite structure. Throughout the valve there are scattered collection of cells, lymphocytes, histiocytes, and few polymorphs around vessels with musculo-elastic walls (Plate XXX, fig. 18). *Auricular myocardium :* Fibres show marked hypertrophy.

Section III. (P. P. M.) Shows scattered areas of fibrosis.

Section VI. (T. V.) Valve distorted and shows an organizing vegetation with numerous endothelial-lined spaces (Plate XXX, fig. 17). *Valve ring :* Vascularized—the vessels show musculo-elastic walls with sparse collection of lymphocytes. The entire structure of the valve presents a hyaline structure with scattered collection of lymphocytes, histiocytes, and occasional polymorphonuclear leucocytes. *Myocardium :* The fibres show hypertrophy.

DISCUSSION AND COMMENT.

All the hearts were heavier than normal. The average weight of Indian hearts is, males 7½ oz. and females 6 oz. (Buchanan and Maddox, 1902). The right auricle was dilated in two cases and the left auricle was dilated in one and dilated and hypertrophied in two cases. The right ventricle was dilated and hypertrophied in three cases and the left ventricle was not markedly hypertrophied in any case.

The pericardium showed signs of inflammation of varying degree in all the four cases of this series. The inflammation was most marked and intense in case I. The pericardial sac was obliterated and organization and marked infiltration with inflammatory cells, mostly lymphocytes was also noticed in this case. The inflammation was minimal in case IV. In case II there was moderate degree of

PLATE XXV.

Fig. 1. Case I. Showing the organizing pericarditis, the loose areolar tissue below and vascular fibrous tissue above. H. & E. (× 100).

R

Fig. 2. Case I. Aortic valve, valve angle and valve ring. Ring spongiosa showing new formed capillary vessels with cell infiltration. The valve angle shows reduplication of the endothelium. (R) H. & E. (× 40).

Fig. 3. Case I. Posterior mitral valve ring showing vascularization and perivascular infiltration. H. & E. (× 160).

PLATE XXVI.

Fig. 4. Case I. Aortic ring showing two arteries with pronounced musculo-elastic hypertrophy and obliteration of lumen. H. & E. (× 40).
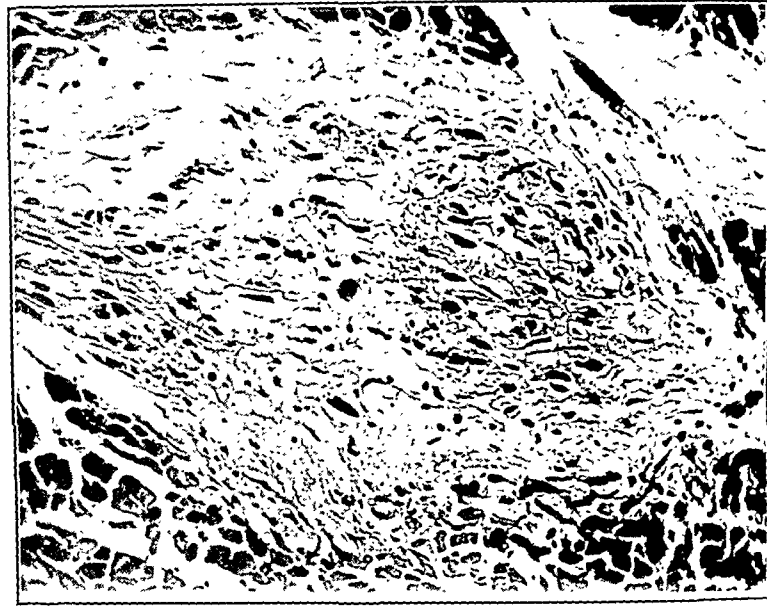
Fig. 5. Case I. A reticular Aschoff body in the muscle of the left vintricle. Note the elongated Aschoff cells and the collagen all round. H. & E. (× 400).

Fig. 6. Case I. A coronal Aschoff body in the muscle of the left ventricle. Note the necrotic reaction in the collagen. H. & E. (× 200).

PLATE XXVII.

Fig. 7. Case I. Pulmonary valve and valve ring showing vascularization and infiltration of the valve ring. H. & E. (× 45).
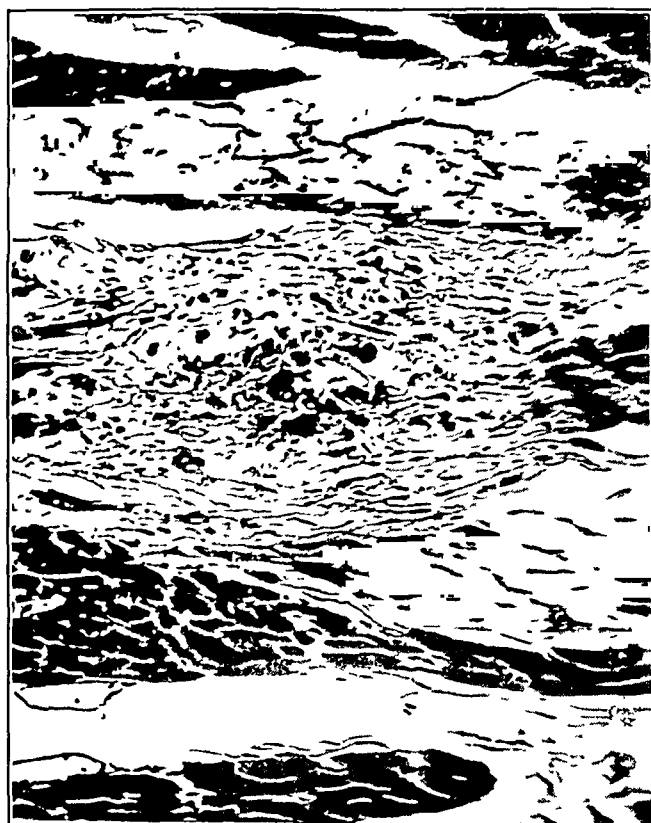
Fig. 8. Case I. A coronal Aschoff body under higher magnification showing cell details. H. & E. (× 400).

Fig. 9. Case I. A coronal Aschoff body in the left posterior papillary muscle. Note the para-arterial situation. H. & E. (× 100).

PLATE XXVIII.
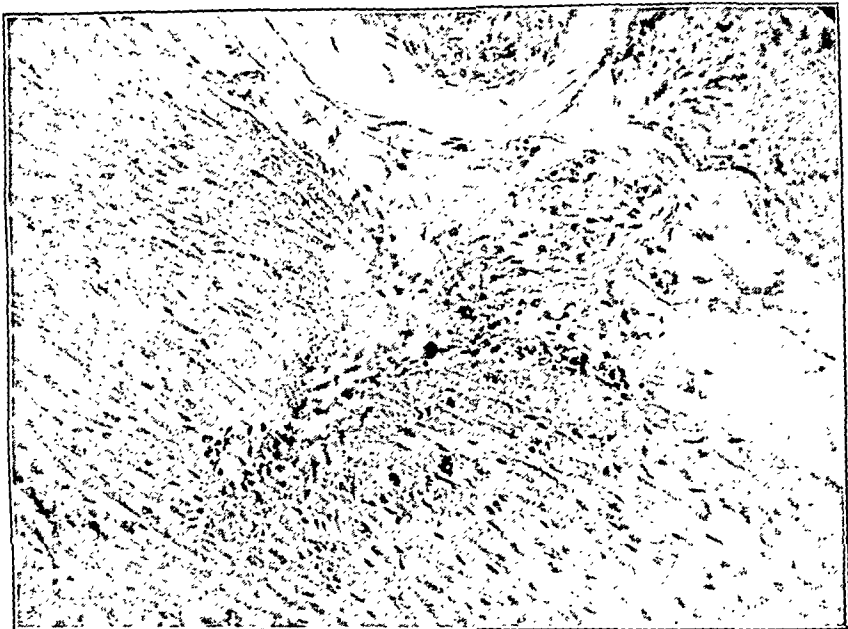
Fig. 10. Case III. Sub-pericardial infiltration breaking through the muscle of the left ventricle. H. & E. (× 200).

Fig. 11. Case II. The tricuspid valves showing sclerosis and granular vegetations near the free margin. Note the vegetations on the chordæ tendineæ and tips of papillary muscles.

Fig. 12. Case II. Button-hole mitral opening showing a row of granular vegetations on the inner aspect.

PLATE XXIX.

Fig. 13. Case II. An atypical fibrillary Aschoff body in the interventricular septum. H. & E. (× 200).
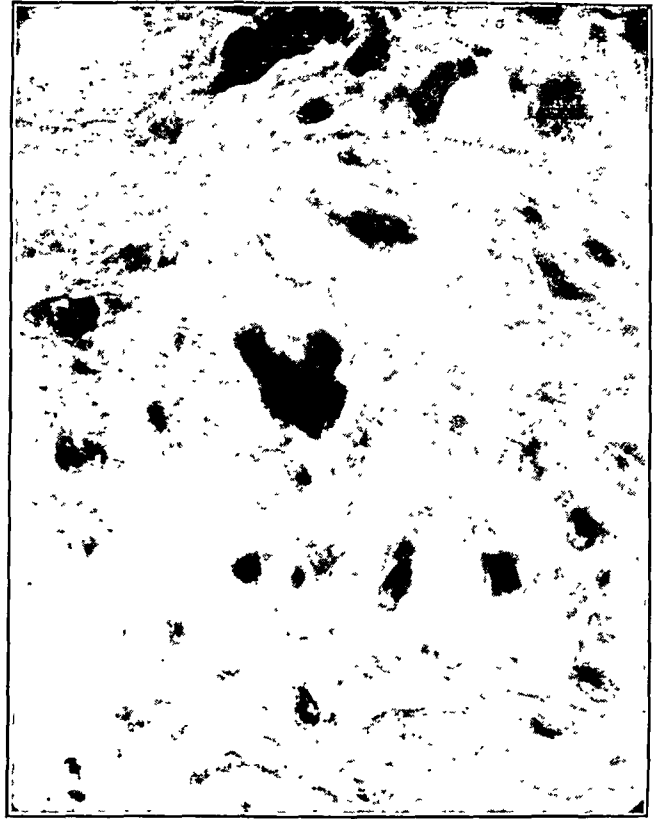
Fig. 14. Case II. Multinucleated Aschoff cells in the posterior mitral valve. H. & E. (× 600).
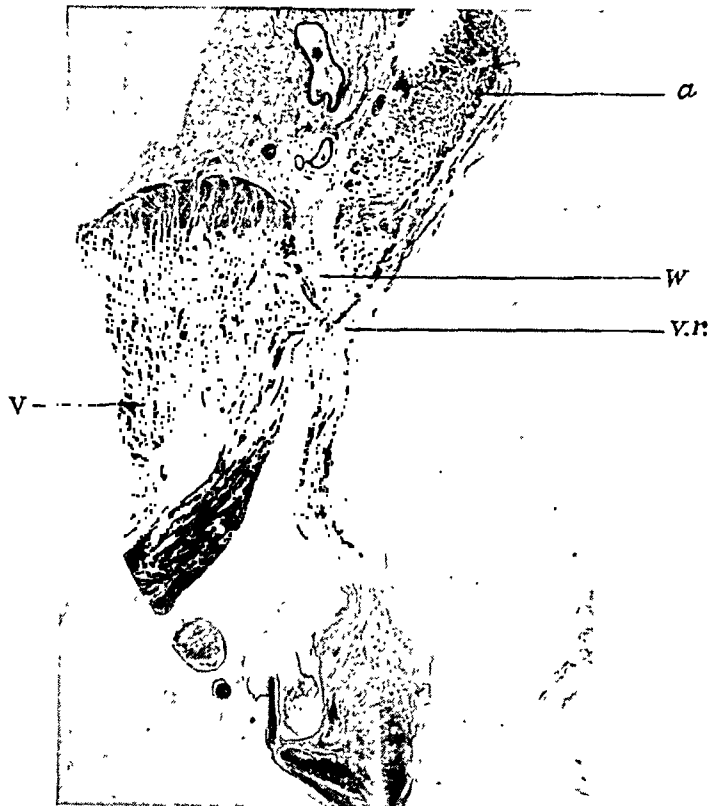
Fig. 15. Case II. Section of posterior mitral valve showing the left auricle (a), the left ventricle (v), the intervening pericardial wedge (w), showing the mode of spread into the valve ring (v.r.). H. & E. (× 4).

PLATE XXX.

Fig. 16. Case IV. Section of posterior mitral valve showing the deformity and hyaline fibrosis of the valve. H. & E. (× 6).

Fig. 17. Case IV. The septal tricuspid valve showing deformity and hyaline fibrosis and two organizing vegetations (v). H. & E. (× 5).

Fig. 18. Case IV. Two vessels in the spongiosa of the posterior mitral valve showing musculoelastic changes and perivascular infiltration. H. & E. (× 140).

inflammation and few petechial hæmorrhages were found on the surface of the pericardium. Effusion occurred only in one case, case III. This pericarditis is of great interest as clinically, evidence of this inflammation is not commonly met with in our hospital practice, as it is latent in most cases. The pericardium is wedged in between the auricles and ventricles and the big vessels and this pericardial wedge plays an important rôle in the spread of the rheumatic infection to the other parts of the heart. This wedge is in close proximity to most of the rings of the valves particularly the posterior mitral ring. The ring of this valve was found to be vascularized and infiltrated with inflammatory cells in all the four cases of this series. The ring changes were most intense in case I and in this case the pericarditis was most marked. The ring being invariably inflamed, the inflammation may spread from it to the pericardium via the ring to the wedge or the inflammation may spread from the pericardium via the wedge to the ring. In one case (case III) the pericardial infiltration could be demonstrated breaking its way into the ventricular myocardium. It is possible that this mode of spread also occurs in some cases.

Gross and Kugel (1931) have described and defined precisely the topographical relations of certain areas which constitute the proximal portion of each valve leaflet and termed these ' valve rings '. Gross and Friedberg (*loc. cit.*) described the normal features of the various ' valve rings ' and the changes they undergo in rheumatic infection. Normal ' valve rings ' are practically devoid of inflammatory cells. Blood vessels with muscular walls are never seen in the normal ' valve rings '. The incidence of capillaries in the various ' valve rings ' is as follows : anterior mitral ' valve ring ' 1 per cent ; posterior mitral ' valve ring ' 2 per cent ; aortic ' valve ring ' 0 per cent ; tricuspid ' valve ring ' 14 per cent ; pulmonary ' valve ring ' 7 per cent. When present the capillaries are generally few in number, small and circular in cross-section, and easily differentiated from granulation tissue capillaries. In rheumatic infection the ' rings ' are almost invariably infiltrated with inflammatory cells, capillaries, and blood vessels. The latter are sometimes of a characteristic type with musculo-elastic walls. Blood vessels are seldom, if ever, present in normal human heart valves. The ' valve ring ' must be considered a strategic site in the pathogenesis of rheumatic valvulitis. The aortic and the posterior mitral ' valve rings ' are particularly important as they are normally free from capillaries and devoid of inflammatory cells and any change in them afford very valuable information with regard to rheumatic infection. All the four cases in this series showed changes in the ' valve rings '. The vascularization and infiltration with inflammatory cells was very pronounced in case I in the aortic, posterior mitral, tricuspid, and pulmonary, ' valve rings '. Case II showed vascularization and moderate infiltration with inflammatory cells of the posterior mitral and the tricuspid ' valve rings '. In cases III and IV scarring was the marked feature of the posterior mitral and tricuspid ' valve rings ' and the infiltration with inflammatory cells was not so very pronounced as in the first two cases. In none of the four cases of this series was the inflammation confined to the rings only. The changes had spread to the corresponding valves. The valvular changes showed much variation. In case I there was no gross distortion of any of the cusps. The endocardium of the cusp was thickened and opaque and the valves themselves appeared thicker than normal. In cases II and III, there was well-marked mitral stenosis and the tricuspid valves were thickened and opaque. In case IV there was both mitral and tricuspid stenosis

and the valve cusps were distorted and fibrosed. The spongiosa was thickened and the deeper layers were vascularized and infiltrated with inflammatory cells in three cases of this series. In case II, Aschoff bodies were found in the posterior mitral valve in the deeper layers of the spongiosum. In case IV the bulk of the posterior mitral and septal tricuspid valves was converted into hyaline masses without any definite structure. Throughout the valves—posterior mitral and tricuspid septal cusp—there were scattered collection of cells, around vessels with musculo-elastic walls. The cells were lymphocytes, histiocytes, and few polymorpho-nuclear leucocytes.

Vegetations were found in three out of the four cases of this series. In the first case they were found on the aortic valves. In two (cases II and III) they were found on the tricuspid valves and in one (case II) on the margins of the slit-like mitral opening. Only in one (case II) vegetations were found on few of the chordæ tendineæ and papillary muscles.

The valvular changes in case IV are very instructive. Naked-eye examination showed marked fibrotic changes both in the endocardium of the auricle and in the auriculo-ventricular valves. The proximal portions of the mitral valve-cusps were thrown into prominent ledges. The distal portions of the mitral and tricuspid valves were thick and deformed. There was stenosis of the mitral as well as the tricuspid opening. Vegetations were found on the margins of the tricuspid opening. On microscopic examination these vegetations were found to be organized. The whole picture was suggestive of healed rheumatic infection. Is it possible that these permanent cicatricial deformities are ever established without the intervention of an acute stage ? The histopathological evidence reveals this possibility as unlikely. It showed the ' valve rings ' to be vascularized and infiltrated with, not only inflammatory cells, but also plasma cells and histiocytes. The vessels showed musculo-elastic walls both in the posterior mitral and tricuspid ' rings ' and in the depth of the posterior mitral and the septal tricuspid valves. In the discussion of the changes in the ' valve ring ' and valves, it has been shown, the above findings are evidences of rheumatic activity. But there was no rheumatic history in this case. There is the possibility that occasionally these valvular changes are produced by an unbroken sequence of sub-acute rheumatic infection. This view has been advanced by Stott (1930). The evidence adduced above makes it more probable that even in these cases there must have been phases of activity, the histological counterpart of which must be the acute valvulitis revealed by the microscope, which at least lays the foundation of that progressive fibrosis that ultimately leads to the gross changes which end in the valvular deformity and stenosis. This would explain the common finding of both active and obsolete changes in the same valve at the same time.

In case I, Aschoff bodies were found in the posterior wall of the left ventricle (M.P.), the interventricular septum (T.V.), and the left posterior papillary muscle (P.P.M.), in the order of their frequency. The left ventricle showed numerous Aschoff bodies some in the muscle and the rest in the intermuscular connective tissue. They differed very much in their structure. Some of them showed numerous giant cells, while others were more fibrillar. Gross and Ehrlich (1934) in their studies on the Aschoff bodies have described seven types of these and they have also correlated these various types with the clinical course of the disease. The

reticular. small and large cell coronal, mosaic and polarized types were found. In the septum, the polarized and fibrillar types were found but they were not so numerous as in the left ventricle. In the posterior papillary muscle the reticular and coronal types were found and they were still fewer in number. In case II where also a history of an acute attack prior to the final one was obtained, Aschoff bodies of the fibrillar type were found in the interventricular septum (T.V.) only. In cases III and IV Aschoff bodies were not found in the myocardium. Case III showed remarkable changes in the myocardium and the coronary arteries. In the posterior wall of the left ventricle. the branches of the coronary artery were dilated, tortuous and showed thickening of the periarterial connective tissue with occasional fibrocytic cell collection. In the interventricular septum the same changes were noted in the branches of the coronary artery and some of them showed marked endothelial and sub-endothelial proliferation. The fibrosis was very pronounced and extensive. In places it invaded the myocardium converting it into an ill-defined and poorly-staining mass. Sparse round and fibrocytic cell infiltration was noted throughout the myocardium. In case IV some of the branches of the coronary artery showed very pronounced endothelial and sub-endothelial proliferation. The periarterial fibrosis was not very pronounced. In the two cases discarded, no Aschoff bodies were found in the myocardium. Gross and Ehrlich (*loc. cit.*) give the incidence of Aschoff bodies in cases showing evidences of activity, clinical or pathological. as 90 per cent and in others as 56 per cent. The number of cases studied in this series is too few to draw any conclusion, but compared with their figures the incidence of Aschoff bodies in our material is much lower and works out about 33 per cent.

The findings in the myocardium considered along with the findings in ' the valve rings ' and the valves, bring out some very interesting facts. Taking as the anatomical evidences of activity fresh verrucous lesions, fresh pericarditis, acute inflammatory phenomena in the myocardium, ' valve rings ' and valve leaflets, all the four. cases must be classed as active. The evidences of activity in the four cases though varying much in degree were nevertheless definite. Yet only in the first two cases Aschoff bodies were found. These were active cases both clinically and pathologically. In cases III and IV no Aschoff bodies were found in the myocardium, while the ' rings ' and the valves showed evidences of rheumatic activity. This absence of Aschoff bodies in cases III and IV in which there was no clinical evidence of activity but only a pathological one, raises the question of the possibility of permanent cicatricial deformity like mitral stenosis being established without the intervention of a clinically acute state. In other words is it possible to have an insidious form of cardiac rheumatism akin to that of the more active rheumatic condition, in its nature, its distribution, and its ultimate results, differing only in its more protracted nature as if it has been produced by a long series of diluted doses instead of by a short series of concentrated doses ? This view has been advanced by Coombes (*loc. cit.*). The present study lends support to this contention. In cases III and IV and also in the two cases discarded there was well-marked mitral stenosis but there was no rheumatic history. Histologically cases III and IV showed evidences of activity in the ' valve rings ' and valves and yet no Aschoff bodies were found in the myocardium. The effects of rheumatic infection were confined to the endocardium, the valves and the endothelium of the artery. This can be explained by the assumption of Coombes that the infective agent enters the heart

in doses too small, too thoroughly diluted, to provoke any perivascular reaction at all ; yet the inner wall of some of the coronary twigs at any rate is so frequently reached and irritated by the infective agent, small though the dose may be, that it always shows in every case of cardiac rheumatism, a definite proliferative re-action. Even when it is strong enough to provoke any perivascular reaction, it provokes only a diffuse perivascular fibrosis which may slowly invade the myocar-dium and even replace it in some places as in case III, it is not strong enough to evoke the formation of Aschoff bodies in the muscular walls of the heart. The material studied is too small to dogmatize on this, but further studies in this direction would throw much light on one of the most vexed problems of cardiac rheumatism as it is met with in a majority of cases in South India.

Summary.

1. A brief summary of the available Indian medical literature on the subject is given.

2. The importance of examining blocks cut from definite topographic sites in the heart according to the standardized technique of Gross, Antopol and Sacks in the study of cardiac rheumatism is emphasized.

3. The morbid anatomy and histopathology of four rheumatic hearts are described in detail.

4. The rôle played by pericarditis and the pericardial wedge in the spread of rheumatic infection in the heart is discussed.

5. The strategic importance of the ' valve ring ' in the pathogenesis of rheuma-tic valvulitis is discussed.

6. The possibility of permanent circatricial deformities in the valve being established without the intervention of an acute stage is discussed in the light of the histopathological evidence presented.

7. The differences in the histopathological changes in the myocardium and the valves between cases which were clinically and pathologically active and cases which were only pathologically active are discussed.

8. The possibility of the existence of an insidious form of cardiac rheumatism with evidence of activity in the ' valve rings ' and valves only and without Aschoff bodies in the myocardium is discussed.

9. Microphotographs to illustrate the histopathology of rheumatic heart disease are given.

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DIPHTHERITIC ULCERS OF THE SKIN.

THE 'GARIGHA' OF CHITTAGONG HILL TRACTS.

BY

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KNOWLES AND FRESCOLN (1914) summarized the literature bearing on diphtheria of the skin and recorded two patients in whom the skin lesions were of the bullous impetigo type. Rogers (Rogers and Megaw, 1939) in describing the ætiology of Veld sores writes that some of the ulcers are caused by the diphtheria bacillus and that in such cases antidiphtheritic serum is an effective form of treatment. A more detailed description is given by Manson-Bahr (1935) who states that a certain proportion of Veld sores is diphtherial in origin caused by the toxigenic diphtheria bacilli. This author records that typical diphtheritic paresis or paralysis has been observed in association with these sores (in one series this complication occurred in 27 per cent of cases), and that certain outbreaks are associated with faucial diphtheria. This opinion is expressed by the majority of the writers on skin diseases. Barber and Knott (1920) described a case of chronic ulceration of the skin involving the wrist and the left leg in a soldier which was due to infection with an atypical diphtheria bacillus. This case is also referred to by Forman (1937) and Forman and Koerner (1939). The sugar reactions of the organism isolated from the ulcer were closely allied to those of *Corynebacterium diphtheriæ.*

In India Bensted (1936) described an outbreak of diphtheria amongst soldiers during military operations in the North-West Frontier Province. In this outbreak both faucial and cutaneous lesions were noted. The skin ulcers, locally known as

'Frontier sores', followed trivial injuries received during climbing up or scrambling down the steep rock-strewn hill-side. The typical picture was that of a deep punched-out ulcer with a thick unhealthy edge and with a floor covered with a dirty grey membrane that contained an almost pure culture of *C. diphtheriæ*. The ulcers had a fœtid odour and were slow in healing. In most cases there was an almost dramatic response to the administration of diphtheria antitoxin. Thirty-one cases of ulcers of the skin were seen by this author who carried out extensive and painstaking laboratory investigations. Two of the patients with skin lesions were found to be suffering from mild faucial diphtheria. In view of the fact that the strains isolated from these cases were toxigenic and that paresis occurred in some of the cases and that faucial diphtheria was present at the time, this author came to the conclusion that, whilst certain cases of the sores may originate as a direct infection with diphtheria bacilli, it would appear unlikely that this was frequently the case. Further, that it was not sound to assume that *C. diphtheriæ* was of great importance as a primary ætiological agent in the causation of frontier sore although as a secondary invader, it must be treated with all seriousness.

In the present paper are recorded the results of investigation carried out in five patients suffering from ulcers on the feet or on the lower part of the leg. *C. diphtheriæ* was isolated from each of the 21 ulcers present in these five patients and typical ulcers were reproduced in man by the injection of the organism isolated from these patients.

In August 1938 two patients and in August 1939 three patients suffering from indolent ulcers of the legs were sent from Rangamati, Chittagong Hill Tracts, to the Carmichael Hospital for Tropical Diseases, Calcutta. From information supplied by the Civil Surgeon, Chittagong Hill Tracts, and the patients themselves the following points are extracted: These sores are locally known as 'Garigha' ('wheeled' sores) and occur most frequently in the rainy season. A few cases occur each year during the monsoon rains but occasionally the disease assumes an epidemic form when many individuals are affected. The last big outbreak is said to have occurred in 1921. The sores affect both hillmen and plainsmen. The males are more often affected than the females, and those engaged in outdoor manual work suffer more often than indoor workers. No age is exempt, babies in arms occasionally develop the sores. The sores have never been associated with outbreaks of faucial diphtheria or paresis. One attack does not confer any immunity and re-infection through another superficial scratch has been often noted.

The disease is characterized by painful ulceration on the exposed parts of the body, most commonly on the lower limbs. The infection appears to take place through some abrasion or scratch which becomes inflamed and gradually breaks down to form ulcers. The ulcers vary in size from $\frac{1}{4}$ inch to $1\frac{1}{2}$ inches in diameter. In some cases the ulceration is more extensive by the coalescence of neighbouring ulcers. There is no fever or any constitutional disturbance but there is severe pain and the sufferer is unable to attend to his work. The ulcers are slow in healing and take from 2 to 3 months to heal. The outbreak subsides with the cessation of the monsoon rains. The disease has never been known to lead to any serious after-effects but the economic loss resulting from the incapacity to work in the fields during the monsoon rains is a matter of serious concern.

The five patients were young well-built hillmen between the ages of 20 and 35 years. There was a history of injury in each of the patients, in one a leech-bite, in the second a thorn-prick, in the third a local injury from an ill-fitting boot, in the remaining two slight scratches on the feet received during a game of football played bare-footed. The initial injuries were trivial in nature and healed up in 2 or 3 days but after an interval of 10 to 15 days a small vesicle full of slightly turbid fluid developed at the site of the original injury. This soon broke down to form a small ulcer. At this time there was considerable pain and the patients were unable to move about without great discomfort. The ulcer gradually increased in size and after 7 to 14 days reached its maximum size. The healing was slow and when healed there remained a firm depigmented scar (Plate XXXI, fig. 1). Throughout the course there was no rise of temperature, no enlargement or tenderness of the regional lymph glands, or any other constitutional disturbance.

In all the five patients the ulcers were on the foot or on the lower part of the leg. In two patients the ulcers were single, large, and oval in shape. In the third patient there were three ulcers (1 large and 2 small), in the fourth patient 5 ulcers (2 large and 3 small), and in the fifth patient 11 ulcers (3 large and 8 small). The ulcers in the fourth and fifth patients were on both legs. The large ulcers were round or oval and 1 inch to $1\frac{1}{2}$ inches in diameter. The smaller ulcers were $\frac{1}{8}$ to $\frac{1}{2}$ inch in diameter (Plate XXXI, fig. 2). When first seen the majority of the ulcers were in a dirty condition, some were covered with a leaf or other unsatisfactory dressings. After a few simple saline dressings the ulcers became clean and all the ulcers, whether small or large, were of the same general type. The ulcers were punched-out with somewhat thickened and undermined edges. The base of the ulcers was covered with an unhealthy and feebly-developed granulation tissue with a few easily removable flakes of pus but the diphtheritic false membrane was not seen in any of the ulcers. A little pus could be pressed out from under the margin. The ulcers bled readily when touched moderately heavily with a platinum loop. There was slight induration around the ulcers except in one patient who had six ulcers situated in an area of about 3 inches in diameter in front of the lower part of the leg in whom there was considerable degree of induration in the surrounding skin (Plate XXXII, fig. 3). The ulcers were exceedingly tender to touch.

The five patients were observed for a period of 1 to 3 months. In all except one patient who had an attack of malaria, the temperature and pulse were normal, appetite was good, and but for the pain caused by the ulcers the patients were well. All the five patients had heavy hookworm infection and one had *Entamœba histolytica* infection. The Wassermann reaction was negative in all.

Smears of pus from the base or from the undermined edge showed pus cells, Gram-positive bacilli, and cocci.

Many of the pus cells were crowded with bacilli. Several direct films of pus and exudate were examined for the presence of fusiform bacilli, acid-fast bacilli, amœbæ, leishmania, fungi, and yeasts. None of these organisms were seen in more than 60 smears examined from the 21 ulcers present in the five cases. Spirochætes were not seen in stained smears from the exudate or in wet films examined under dark-ground illumination.

Pus from the undermined edge of the ulcer was inoculated on nutrient agar, 5 per cent sheep's blood agar, and on tellurite blood agar, and incubated aerobically at 37°C. Other sets of nutrient-agar and blood-agar plates were inoculated with pus and incubated anærobically in the presence of hydrogen (MacIntosh and Filde's jar) and in the absence of hydrogen (alkaline pyrogallic acid), and under 10 and 20 per cent carbon dioxide. Inoculations were also made on Sabouraud's maltose agar and 1 per cent glucose agar.

From each of the four ulcers on the two patients seen in August 1938 and from each of the 17 ulcers on the three patients seen in August 1939, a non-motile, non-capsulated, non-spore-forming Gram-positive bacillus was isolated. These organisms resembled each other in their morphology and biochemical reactions. They were indistinguishable morphologically and in their biochemical reactions from *C. diphtheriæ.* Other organisms that were isolated but not from each sore included *Streptococcus pyogenes, Staphylococcus aureus* and *albus,* and from one sore a *Corynebacterium* which biochemically resembled *C. hofmannii.* Fungi and yeasts were not isolated from any of the sores.

A brief description of the *Corynebacterium* isolated from the sores is given below :—

Morphologically thin, slender, slightly curved rods, showing marked pleomorphism. There were 2 to 3 granules in the majority of the bacilli. Some bacilli had typical club-shaped ends, some were ' barred ' and some stained uniformly without any bars or granules. The appearance and arrangement of the cells varied with the media on which the culture had been grown. The granules were most marked in cultures grown on Loeffler's serum or on blood agar, whilst long and club forms were more frequent in agar cultures.

On nutrient agar after incubation for 24 hours at 37°C. two types of colonies were seen in some of the strains. The larger colony (1 mm. to 2 mm. in diameter) was circular, somewhat convex with an entire edge ; creamy in colour, moist and glistening with no differentiation and readily emulsifiable in saline. After 4 days' incubation the colony increased to 3 mm. to 4 mm. in diameter with a raised centre and a flat periphery with bevelled margin. The smaller colony which was about 0·5 mm. in diameter after 24 hours' incubation increased only slightly on further incubation. When the small colony was transferred to blood agar the resulting growth could not be differentiated from the growth of the larger colony.

On 5 per cent sheep- or rabbit-blood agar the colonies after 24 hours' incubation were larger (1 mm. to 3 mm.) than the colonies on nutrient agar. The colony characteristics were similar to the growth on nutrient agar. In poured blood-agar plates the deep colonies were tiny and elongated with tapering ends resembling those of *Streptococci* but somewhat larger in size. There was a small zone of β type of hæmolysis round each colony. No intact red-blood cells were seen in the hæmolytic zone.

On tellurite blood-agar plates after incubation overnight, there were convex, smooth, shiny black colonies with an entire edge which on further incubation increased in size and were differentiated into a raised centre and flat periphery.

On Loeffler's serum there was a good growth which was creamy in colour. There was no liquefaction of the medium. On nutrient-agar slope a good growth

was obtained which was faintly orange in colour. The growth on agar was distinctly more profuse than that obtained with laboratory strains of *C. diphtheriæ.*

In broth after 24 hours' incubation there was a diffuse moderate growth, with a slight non-granular deposit. There was no surface growth. Better growth was obtained in 10 per cent serum broth.

On potato, the growth was not visible to the naked eye. In semi-solid agar there was a scanty growth along the tract of the stab.

The optimum growth was obtained under ærobic conditions, but growth also occurred under complete anærobiasis.

All strains gave similar biochemical reactions. They fermented glucose, maltose, dextrin, and galactose, with the production of acid but no gas. Lactose, saccharose, dulcite, mannite, and inulin, were not fermented and there was no appreciable change in litmus milk. There was no change in starch and glycogen. Indole was not formed. Nitrates were reduced. Filtrable hæmolysin (for rabbit red-blood cells) was present in filtrates of 24 or 48 hours' old serum-broth cultures.

No exotoxin was present in filtrates of 7 days' old cultures of the organism in Hartley's broth. When injected intramuscularly or intraperitoneally this organism did not kill a guinea-pig or a rabbit even when very large doses were used. When injected intradermally it caused local flush and an inflammatory nodule which was retrogressive in character and subsided in 3 or 4 days. Injected intradermally in a monkey, there was local œdema and redness which subsided without any ulceration in four to six days. There was no invasion of the tissues by this organism. Injected into the pectoral muscles of a pigeon the organism produced no appreciable change. Young culture of the *C. diphtheriæ* isolated from the ulcers was smeared on the lightly-abraded conjunctiva, the mucous membrane of the nose and the vulva of guinea-pigs. No lesions were produced at any of these sites. Control inoculations with a freshly isolated strain of toxigenic *C. diphtheriæ* and the standard strain Park 8 produced typical membrane-formation with local œdema and later death of the animals.

Many experiments were carried out in man. A general summary of the results is given below :—

(1) Pus from the sores when inoculated by a scratch produced a typical ulcer in five days. *C. diphtheriæ* was recovered from the ulcer. When kept clean this ulcer healed up in 10 to 14 days.

(2) Filtrate of the pus from the ulcer injected intradermally produced no effects (Plate XXXII, fig. 4-A).

(3) When a culture of the *C. diphtheriæ* isolated from the ulcers was injected intradermally there was local redness in 24 hours, a pustule developed in 3 to 4 days which broke down to form an ulcer in 5 to 6 days. The inoculated organism was recovered from the ulcer in pure culture (Plate XXXII, fig. 4-C).

(4) *C. diphtheriæ* and filtrate of the exudate from the sores when injected intradermally gave similar but somewhat more marked results than those obtained with the organism alone (Plate XXXII, fig. 4-B).

(5) Intradermal injection of a mixture of the *C. diphtheriæ, Staphylococcus aureus,* and *Streptococcus pyogenes* produced marked local reaction and an ulcer in 5 to 6 days (Plate XXXII, fig. 4-D).

(6) Culture of a diphtheroid isolated from one of the patients produced no effects when injected intradermally (Plate XXXII, fig. 4-E).

(7) Intradermal injection of *Strept. pyogenes* isolated from the sore produced minimal local reactions which subsided in a few days.

(8) Attempts to produce infection without preliminary injury to the surface epithelium were not successful. The simple application of cultures of bacilli to the skin was without results.

The above experiments were carried out on individuals suffering from the sores. No appreciable difference was observed in the reactions obtained in individuals with recently-formed ulcers and in persons in whom the naturally-acquired ulcers had healed. In all there was a slight but transient rise of temperature, a feeling of general malaise and headache but no enlargement of regional lymph glands.

Phenolized heat-killed suspension of the *Corynebacterium* isolated from the ulcers when injected in doses of 15 million organisms gave severe local reactions (marked œdema, redness, and tenderness) which subsided within a week.

Three of the five patients were tested for the Schick reaction and were Schick-negative. The diphtheria antitoxin in the serum of three patients (employing a standard antitoxin and using the skin reaction in a guinea-pig) was found to be approximately 1/20 unit of antitoxin.

Agglutinins for the *Corynebacterium* isolated from the sores were present in the serum of the three patients examined this year. The serum agglutinated the strain isolated in 1938 and the strain isolated in 1939 and a laboratory strain of toxigenic *C. diphtheriæ.* As agglutination in tubes was unsatisfactory the agglutination test was done on the slide by rubbing a loopful of the culture in different dilutions of the serum. Well-marked agglutination occurred in 1 in 25 dilutions of the patient's serum. There was no agglutination with the diphtheroid or the *Staphylococcus* isolated from the sores. By cross-agglutination with serum raised in rabbits the *Corynebacterium* isolated from the sores in 1938 and in 1939 and the laboratory strain of toxigenic *C. diphtheriæ* were antigenically related. Serum raised with diphtheroids possessed no agglutinins for the other organisms.

Injections of live cultures of the *Corynebacterium* isolated from the sores into a healed sore reproduced a small ulcer in 5 to 6 days. There is thus no local immunity.

Swabs taken from other areas of the skin of the patients suffering from the ulcers did not yield any *Corynebacteria* resembling the *Corynebacterium* isolated from the sores.

C. diphtheriæ was not isolated from the throat and nasopharynx of the five patients suffering from ulcers. Material taken from ulcers showing definite signs of healing was examined for the presence of bacteriophage active against the organisms isolated from the ulcers. No bacteriophage was found even after repeated passage of the filtrate of the material on young cultures of the organisms.

PLATE XXXI.
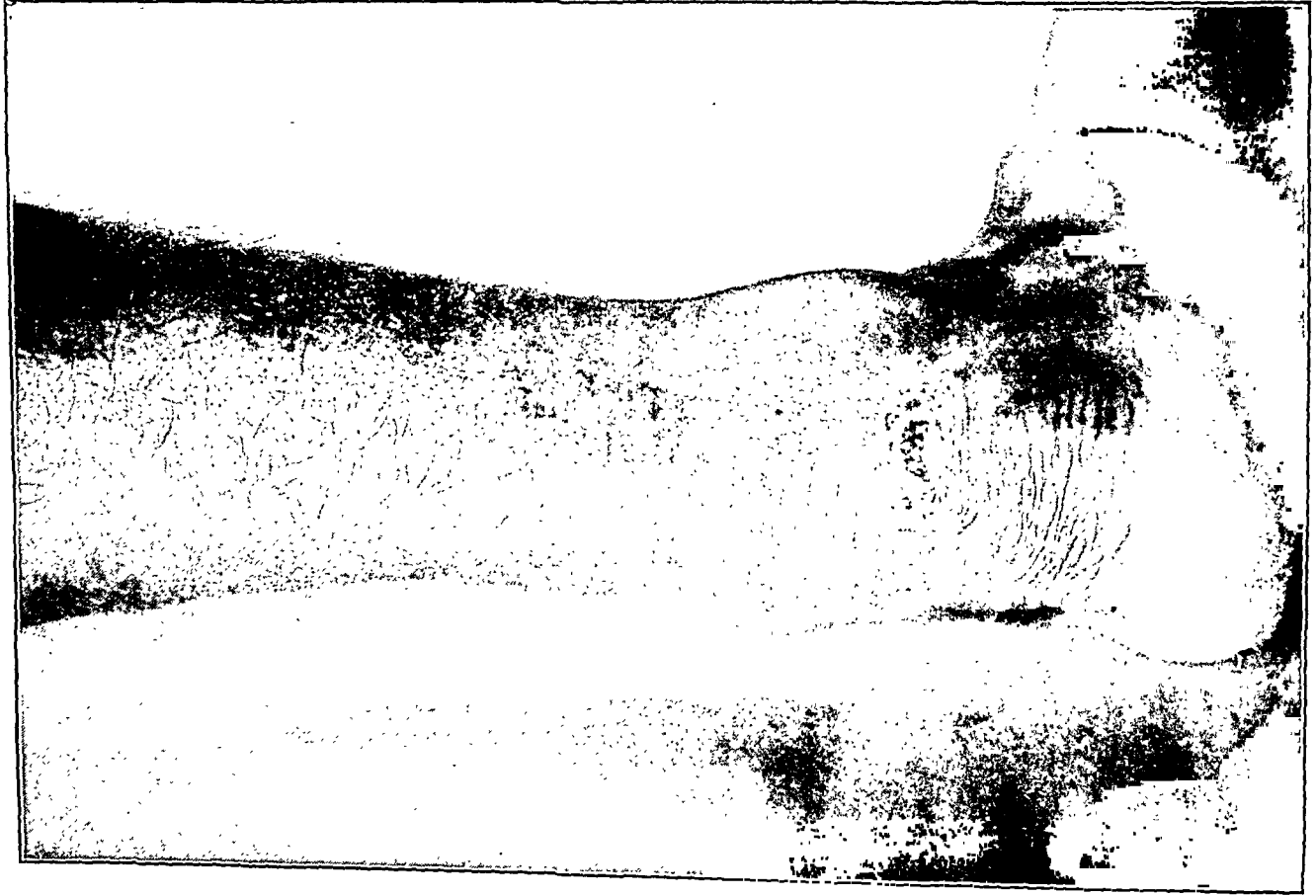
Fig. 1. A nearly healed ulcer and the depigmented scar.
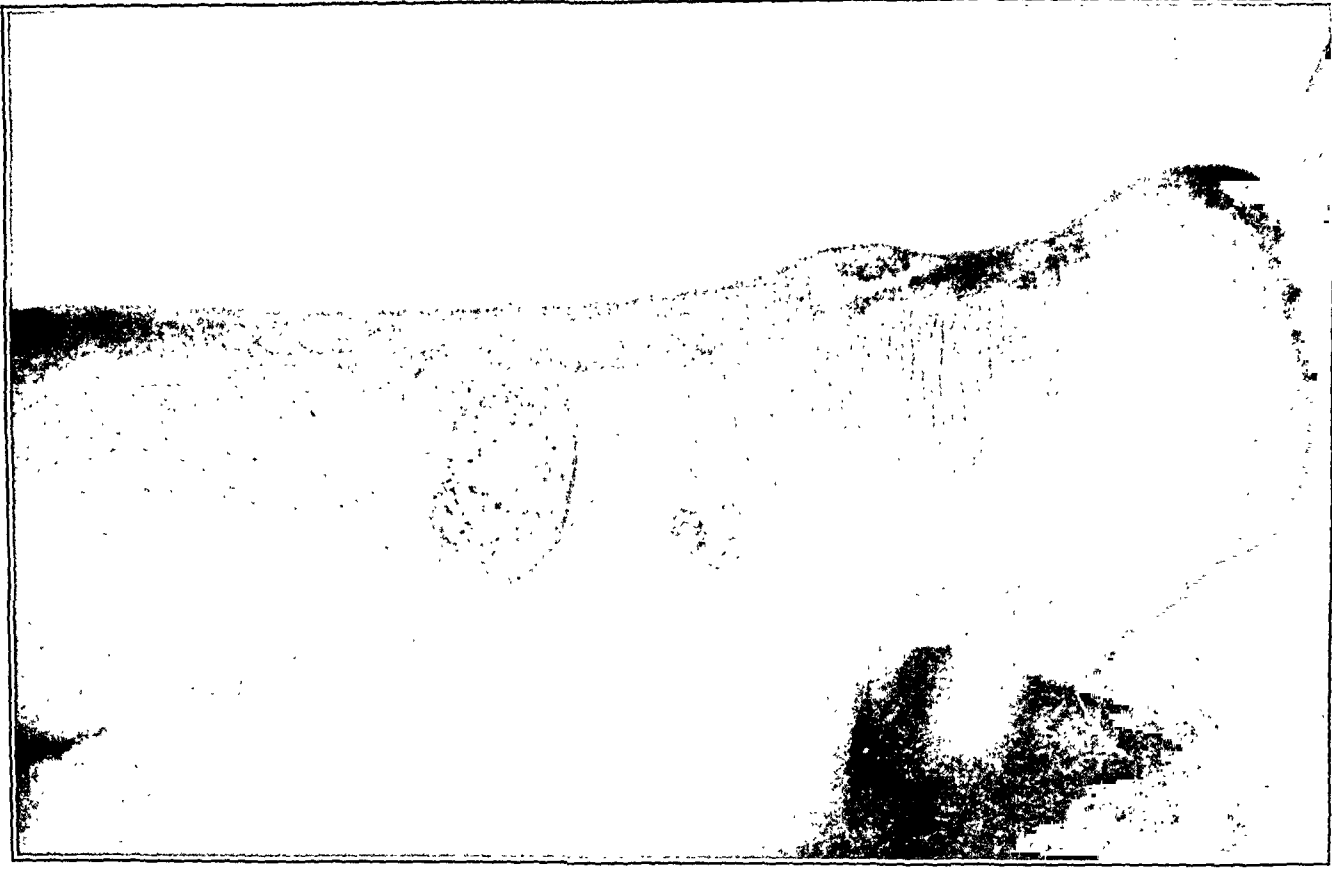
Fig. 2. A large and a small ulcer both of about 2 months' duration.

Fig. 4. The results of experimental inoculation, 4 days after intradermal inoculation of:—

A.—Filtrate of pus from sore.
B.—*C. diphtheriæ* (from ulcer) plus filtrate of pus.
C.—*C. diphtheriæ* alone.
D.—*C. diphtheriæ* with other organisms isolated from the natural sore.
E.—Diphtheroid culture.

Fig. 3. A group of ulcers on the leg and a single ulcer on the dorsum of the foot near toes.

A small wedge-shaped piece of tissue was removed from the edge of the ulcer, fixed in Zenker's, cut in paraffin, and stained with hæmatoxylin and eosin. There was complete loss of epidermis and there was extensive cellular infiltration of the dermis. The cellular elements present were mainly of the mononuclear types, young fibroblasts, and red blood corpuscles. There were no degenerative changes typical of diphtheritic lesions nor the formation of any fibrinous exudations. There were no plasma cells, epithelioid cells or giant cells. There were signs of inflammation in the area adjacent to the ulcer.

Antidiphtheritic serum which has been reported to produce a striking effect in the healing up of diphtheria ulcers of the skin (Veld sores, Frontier sores, etc.) was found to be without any effect in these patients. The serum was given subcutaneously in the vicinity of the sore, injected intramuscularly, and also used as a dressing (lint soaked in serum) but without any effect. The dose of antidiphtheritic serum injected was 8,000 to 12,000 units.

'M. & B. 693' was given by mouth to one of the patients for 5 days without producing any appreciable effect.

Various antiseptic dressings, such as potassium permanganate lotion, gentian violet (5 per cent), carbol fuchsin (1 per cent). hydrogen peroxide, were no more efficacious than simple saline dressings, once the gross infection had been eliminated.

Vaccine prepared from the organism isolated from the sores appeared to have a favourable effect, the ulcers healing up in 2 to 3 weeks.

The fact that from two outbreaks, one in August 1938 and one in August 1939, identical strains of *Corynebacterium* were isolated is by itself of significance. These strains resemble each other and correspond fully with the diphtheria bacillus in their morphological, cultural, and fermentative characters but are not toxigenic. They must therefore be regarded as non-toxigenic diphtheria bacilli. Repeated microscopic examinations of the pus did not reveal any fusiform bacilli, fungi, or yeasts. Cultural examinations carried out on different media and under different atmospheric conditions yielded the *Corynebacterium diphtheriæ* with regularity. Other organisms, such as *Strept. pyogenes*, *Staph. aureus* and *albus*, were found occasionally in varying numbers and combinations. Injection of living *C. diphtheriæ* into man reproduced typical ulcers and from these ulcers the organism was again isolated. This was done on three of the five individuals suffering from the sores. Freshly-isolated strains gave rise to marked reactions resulting in the formation of ulcers, whereas old cultures tended to produce retrogressive lesions. There is sufficient experimental data to justify the conclusions that these ulcers are caused by a non-toxigenic strain of *C. diphtheriæ*.

SUMMARY.

Five patients suffering from ulcers on the legs or feet have been studied. These ulcers occur in Chittagong Hill Tracts and are locally known as 'Garigha' or 'wheeled' sores. A few individuals are affected each year during the monsoon rains. Occasionally the disease assumes an epidemic form when many persons are affected. No age or sex is immune. The infection takes place through some

injury or scratch which becomes inflamed and then breaks down to form painful ulcers which are slow in healing.

Two patients were seen in 1938 and three patients in 1939. From each of the 21 ulcers in the five patients one organism was regularly and repeatedly isolated. This organism resembled *C. diphtheriæ* in its morphology and biochemical reactions, but was not toxigenic. An extensive study of this bacillus and comparisons with the characteristics of the species in the genus *Corynebacterium* listed by Bergey (1939), and Topley and Wilson (1936) place this organism as non-toxigenic variant of *C. diphtheriæ*. According to the character of growth on tellurite blood agar and in liquid cultures, and in its inability to attack starch or glycogen and in being hæmolytic this organism belongs to the mitis type of *C. diphtheriæ*. Other organisms, such as *Strept. pyogenes*, *Staph. aureus* and *albus*, were isolated from the sores but with no regularity. All attempts to demonstrate the presence of filtrable virus gave negative results.

The conditions governing the general prevalence of the ulcers and their epidemic rise and fall have not been studied. The contagion is spread by means which are in operation during the rains but the exact circumstances and their incidental co-operation which give rise to epidemic conditions remain to be studied and must be studied in the places where these ulcers occur. Wet weather, however, favour the spread of these sores. This contrasts with the dry desert conditions which are favourable to the spread of Veld sores the causative agent of which has been found by many workers to be the toxigenic *C. diphtheriæ*.

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STUDIES ON RELATIONSHIP OF FLOCCULATION VALUE OF TETANUS-FORMOL-TOXOID DETERMINED BY RAMON'S METHOD AND ITS ANTIGENIC VALUE DETERMINED ON EXPERIMENTAL ANIMALS.

BY

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ANTITETANIC vaccination is now compulsory with the armies of different nations, which signifies a wide acceptance of the value of tetanus toxoid as an immunizing agent against tetanus infection. Future extensive field trials are likely to confirm the correctness of the expectation. as the last Great War confirmed beyond doubt the value of tetanus antitoxin in the prevention of tetanus.

For a fair comparison of the results of different experiments in different countries it is necessary to have a standard for comparing the strengths of various tetanus toxoids used for immunizing different groups of people. For comparing the strength of one lot of toxin with the other, different properties of toxin may be used as criteria. Of these, the minimal lethal dose is the most unstable. and also of no use for comparing two different toxoids. According to Ramon, flocculation value of a toxin is a criterion of its antigenic power at all stages of toxicity. Ramon *et al.* (1937) have shown a fair degree of proportion in the different values, such as toxicity, flocculation value and combining power, of various lots of tetanus toxin, shortly after filtration. Hence any of these values may be used for comparing one toxin with the other. Of these three values, toxicity and combining power cannot be measured in full toxoids. Hence flocculation value only can be used for comparing any two toxoids.

The first question that now arises is whether flocculation value of tetanus toxoid can be determined with so much precision as to make it reasonably reliable. It has been known that flocculation of tetanus toxin-antitoxin combination shows two or more zones. Prevot and Pochon (1938) have recently attributed a combination of flagellar antigen of *Cl. tetani* and its corresponding antibody to be one of the causes of such anomaly. Ionescu-Mihaiesti (1938) considers the cause of occurrence

of multiple zones of flocculation to reside in the antigenic structure of the tetanus bacillus itself and in the presence of flagellar and somatic antigenic components in the toxic filtrate. However, recently Ramon (1938) has brought into notice a special serum which shows only one zone of flocculation with various toxins and is particularly suitable in other ways for the determination of flocculation value of tetanus toxoids. Ramon (personal communication) prepared this special serum No. 678 by immunizing several horses with toxins which showed only one zone of flocculation with antitetanic sera ; and serum from one of them with the said number was found to be particularly suitable for flocculation purposes. Flocculation values of different tetanus toxoids have been determined with the said serum very kindly sent by Ramon, and the results are reported in this paper.

The question that arises next is whether the flocculation value of a toxoid determined by Ramon's method, is a true index of its relative immunizing strength. According to Ramon, there is always a close parallelism between flocculation value and immunizing value of formol toxoids. But Otto and Hetsch (1935) believe differently. They immunized a large number of guinea-pigs with 20 flocculation units in different volumes of three different lots of diphtheria-formol-toxoids containing 3, 11, and 42 flocculation units per c.c. respectively, and found, contrary to expectation, that a toxoid with a lower flocculation value showed a better relative immunizing strength than another with a higher flocculation value. However, they injected different volumes of toxoid keeping unitage constant, while the present method of determining doses of different toxoids is in terms of volume only. It was, therefore, considered interesting to find the immunizing power of a number of tetanus-formol-toxoids in terms of antitoxic response in guinea-pigs injected with the same volume of different lots of toxoids with different flocculation values determined by Ramon's method.

EXPERIMENTAL.

A. Determination of flocculation value.

Different lots of tetanus-formol-toxoids were put in test-tubes in 5-c.c. volumes. Different amounts of special serum No. 678 sent by Ramon diluted so as to contain 300 I. U. per c.c., were put in each test-tube. Flocculation was observed as in previous experiments (Lahiri, 1938). The results are shown in Table I.

Hence the flocculation values of the toxoid lots C.864, 371, 399, 433, and 451 were respectively 39, 6, 9, 24, and 27 flocculation units per c.c. If these figures represent relative immunizing values of those toxoids, then toxoid lot 433 should be four times stronger antigenically than the toxoid lot 371, and toxoid lot 451 should be three times stronger than lot 399, when compared in same volumes. Experiments described under the next headings were intended to determine whether these relationships held true or not.

It is to be noted that there were only single zones of flocculation in each series and flocculations in different mixtures progressed steadily in either directions. Presumably the special serum contains no other subsidiary antibodies against other components in the toxin broth excepting toxin proper.

TABLE I.

Times of flocculation in hours of different toxoid-antitoxin mixtures.

Amounts of serum 678 containing 300 I. U. per c.c.	LOT NUMBER OF TOXOIDS, 5 C.C. IN EACH TUBE.				
	C.864 (Inst. Past.).	371	399	433	451
0·75	2⅙	..	..	..	..
0·70	2	..	..	..	..
0·65	1¾	..	..	..	8
0·60	2½	..	..	7½	6¾
0·55	2½	..	..	6	5½
0·50	2¾	..	..	5	3¾
0·45	3	..	..	4½	3½
0·40	3	..	..	4	4
0·35	3	..	..	6½	7
0·30	3¼	..	..	8	8
0·25	3¼	..	..	..	..
0·20	..	..	10½	..	..
0·15	..	..	9	..	..
0·10	..	**10**	9½	..	..
0·05	..	12	13	..	..

Bold figures indicate initial flocculations.

B. Determination of antigenic value.

Guinea-pigs of from 330 g. to 350 g. body-weight were divided into groups of five. Six such groups were used for immunizing with one lot of toxoid. Four lots

of toxoid numbers 371, 399, 433, and 451 were used for the experiment. Each guinea-pig was injected with 2 c.c. of toxoid subcutaneously.

Twenty-one days later exactly one c.c. of blood was drawn from the heart of each guinea-pig. Bloods from ten guinea-pigs comprising two groups injected with the same toxoid, were pooled in one test-tube. Clotting was prevented by using two c.c. of four per cent citrated saline for ten c.c. of blood from two groups of animals. The mixed plasma of ten guinea-pigs was titrated for antitoxin content. Two more c.c. of the same toxoid were injected subcutaneously on the same day.

One month later, blood from them was drawn in the same amounts, mixed in the same way, and their plasma titrated. The results are shown in Table II :—

TABLE II.

Units (International) of antitoxin in pooled plasma of guinea-pigs immunized with various toxoids.

Groups of guinea-pigs :—	I and II.		III and IV.		V and VI.	
Plasma sample number :—	1	2	1	2	1	2
Toxoid lot 371	>0·5	>12·5	0·5	15	>0·25	>10
$L_f - 6$	<1	<15	..	..	<0·5	<12·5
Toxoid lot 399	>0·1	>5	>0·25	>7·5	>0·25	>5
$L_f - 9$	<0·25	<7·5	<0·5	<10	<0·5	<7·5
Toxoid lot 433	>2	>15	>0·5	17·5	>0·5	20
$L_f - 24$	..	<17·5	<1	..	<1	..
Toxoid lot 451	>1	17·5	1	>17·5	>0·5	>15
$L_f - 27$	<2	..	..	<20	<1	<17·5

DISCUSSION.

All these toxoids were prepared in Veal-peptone-broth medium (Lahiri, 1939) by growing the same strain (A.471 T, from Frankfurt) of *Cl. tetani* for the same

number of days. For detoxication 0·25 per cent commercial formalin was used and the mixtures were incubated at 37°C. to 38°C. for from three to six weeks depending on initial toxicity.

For the experiment, toxoids with lowest and highest flocculation units were selected in order to get sharp differences in results of immunization tests. Differences in the amounts of antitoxin formed as a result of immunization with toxoids of high and low flocculation value were apparent. But they were not exactly proportional to the flocculation values. Moreover, two toxoids—lots 371 and 399—had their flocculation values nearly equal, that of the latter being slightly higher. But the one with the higher value seemed to produce smaller amounts of antitoxin than the one with a lesser value.

Flocculation value of a toxin or toxoid is possibly only a gauge of the intensity and extent of its physico-chemical affinity for its corresponding antitoxin. If it is assumed that antigenicity of a toxin or toxoid, which perhaps depends on the intensity of its capacity to supply stimulus to the antitoxin-producing mechanism, is not dependent on the same component of a toxin or toxoid which determines its physico-chemical affinity with its corresponding antitoxin, then the experimental results can be easily explained. It may be true that generally a toxin or toxoid with high physico-chemical affinity for its antitoxin also possesses a high antigenic quality. Yet dissociation of its qualities may occur, and a toxin or toxoid with high flocculation value and low antigenic value or vice versa, may be expected.

On this assumption it appears that all the different properties of a toxin are functions of different components. A toxin which is not toxic due to inactivation of its toxic component by its interaction with formalin at high temperature may yet be physico-chemically active against its corresponding antitoxin, and antigenic. Similarly, a toxin with a lowered physico-chemical affinity for its antitoxin brought about by some unknown factors, may yet be highly antigenic. Normally speaking, a toxin which is highly toxic may also be highly reactive to its antitoxin and highly antigenic. But in cases of variation, no single property may serve as a gauge of its other properties.

If this assumption is held to be reasonable, then it would appear that the purpose of comparison of immunizing values of any two toxoids may only be best served by testing their antigenic values in a large number of suitable animals, while their flocculation values may serve as a very good guide sometimes giving accurate information about their antigenic values.

SUMMARY.

Flocculation values of different tetanus-formol-toxoids were determined against a special serum prepared by Ramon. Toxoids of different flocculation values were used for immunizing guinea-pigs, and their antitoxic responses were measured in terms of antitoxin unit content per c.c. of circulating blood. It was found that antitoxic response after injection of a toxoid did not always closely correspond with its flocculation value. The possible cause of this variation is discussed.

ACKNOWLEDGMENT.

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TYPHUS IN THE SIMLA HILLS.

Part IX.

LABORATORY OBSERVATIONS, CHIEFLY ON HUMAN XK STRAINS.

BY

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THE PRESENT POSITION.

IT has been recognized for a number of years that the Tsutsugamushi disease of Japan, a variety of the XK serological type of typhus, is conveyed by the larval mite, *Trombicula akamushi*, an ectoparasite of the vole, *Microtus montebelli* (Kitashima and Miyajima, 1918). In Malaya *Trombicula deliensis* found on wild rats is probably the same as *T. akamushi* and is regarded as the vector of rural typhus, an XK serological type of typhus differing from Tsutsugamushi disease only inasmuch as the latter shows primary cutaneous sores and buboes. Apparently the guilt of *T. deliensis* has not yet been definitely proved (Lewthwaite and Savoor, 1936).

An inquiry into typhus in the Simla Hills has been in progress for some years. Typhus strains have been isolated as follows: (1) X19 murine strains from *Rattus rattus* and rat-fleas, and (2) XK human strains from human cases of typhus. So far, no X19 strains have been isolated from human cases and no XK strain has been established either from possible animal reservoirs such as rats or from possible arthropod vectors such as fleas, bugs, ticks, or mites. Rat-fleas are suitable vectors experimentally between rats of the X19 type but there is no definite evidence regarding vectors of the XK type.

Many attempts have been made to isolate XK strains from various sources, especially from mites collected from rats and from mite-infested rats, but without success. In one case an XK strain was apparently recovered from a Trombiculid-infested rat by Dr. R. O. A. Smith but the strain could not be established owing to coincident infections with X19 typhus and *Spirillum minus*.

THE POSSIBILITY THAT MONKEYS ARE IMPLICATED.

On a re-consideration of this work at the end of 1938 it was noticed that little attention had been paid to monkeys, which are in fairly close contact with man and might possibly play a part by harbouring typhus infection and/or attracting and distributing arthropod vectors. An attempt was therefore made to secure some wild monkeys for examination. The first brown monkey, *Silenus (Macacus) rhesus*, which was examined was found to harbour (much to the surprise of the entomologist) a number of larval mites of the suspected species *deliensis*. Altogether six monkeys were available for examination between January and March but only one other yielded a few *deliensis* larvæ. The six monkeys were tested for typhus infection by passage of brain emulsion to guinea-pigs intraperitoneally. There was no positive result although there were a few suggestive febrile reactions. The monkeys then became extremely wily as food was plentiful, and despite regular attempts to trap them the next specimen was not secured until August. It was found to harbour over a hundred *deliensis*-like orange-coloured larvæ, four of which when mounted and scrutinized proved to be actually *deliensis*. Over 100 of these larvæ were emulsified and half of the emulsion was injected intraperitoneally to each of two male guinea-pigs. The results were negative.

The monkey was kept for a fortnight during which the Weil-Felix reaction remained X19 negative, XK + 1 : 25. It was then tested both by citrated blood passage and by brain passage but no evidence of typhus infection was obtained. The results are collected in Table I :—

TABLE I.

Wild brown monkeys, Silenus rhesus. *Attempts to demonstrate natural typhus infection.*

Number.	Date of capture.	Weil-Felix test (XK) X19 neg.	*T. deliensis* larva present.	Result of passage of mites.	Result of passage of monkey brains.
1	27–1–39	+ 1 : 25	20	Negative	Negative.
2	28–1–39	+ 1 : 125	2	..	,,
3	14–3–39	+ 1 : 25	*Nil*	..	,,
4	22–3–39	+ 1 : 25	*Nil*	..	,,
5	22–3–39	+ 1 : 25	*Nil*	..	,,
6	24–3–39	+ 1 : 25	*Nil*	..	,,
7	10–8–39	+ 1 : 50	100 +	Negative.	,,

The grey langurs which are often in the vicinity of habitations are even more elusive and none have been captured.

Conclusion.—The brown monkey, *Silenus rhesus*, is a natural host of the larval mite, *Trombicula deliensis*. Attempts to isolate typhus strains from these monkeys and from larval mites removed from the monkeys were not successful.

Weil-Felix reaction in wild monkeys.

The blood serum of twenty-five wild monkeys from the plains was tested against X19 and XK *Proteus* emulsions with the following results :—

> X19 negative throughout, XK eight + 1 : 50, thirteen + 1 : 25, remainder negative.

Artificial infection of wild brown monkeys, *S. rhesus*, with XK human strains.

Four wild brown monkeys from the plains were injected intraperitoneally with spleen emulsions of passage guinea-pigs which had given typical reactions to infection with XK human strains of typhus. The results were as follows :—

Monkey 1.—Injected with XK human strain Dennison 16th January, 1939. Weil-Felix test weekly, X19 negative, XK + 1 : 25 throughout. P.M. 56th day. Passage of brain to male guinea-pig intraperitoneally—result negative.

Monkey 2.—Injected with XK human strain Dyas 11th April, 1939. Weil-Felix test weekly, X19 negative, XK negative or + 1 : 25 only. P.M. 36th day. Passage of brain to male guinea-pig intraperitoneally—result negative.

Monkey 3.—Injected with XK human strain Dyas 3rd July, 1939. Weil-Felix test weekly, X19 negative, XK + 1 : 25 before infection and one week later, + 1 : 250 after second week, afterwards + 1 : 25 or negative. P.M. 56th day. Passage of brain to male guinea-pig intraperitoneally—result negative.

Monkey 4.—Injected with human strain Dyas 3rd July, 1939. Weil-Felix test X19 negative, XK + 1 : 50 before infection, + 1 : 25 one and two weeks after infection. Died 16th day. Passage of brain to male guinea-pig intraperitoneally—result negative.

Conclusion.—Attempts to inject brown monkeys intraperitoneally with XK human strains of typhus were not satisfactory. Apart from the fact that brown monkeys may harbour the suspected larval mite *T. deliensis*, no evidence has, therefore, been obtained that monkeys are implicated in the epidemiology of typhus, but further work is required.

Persistence of XK typhus infection in passage guinea-pigs.

Guinea-pigs which had been injected intraperitoneally with human XK strains of typhus and which had shown the usual febrile reactions were tested at various

periods after infection by intraperitoneal injection of brain emulsions to fresh guinea-pigs. The results were as shown in Table II :—

<p style="text-align:center">TABLE II.</p>

<p style="text-align:center">*Persistence of human XK typhus in guinea-pigs.*</p>

Strain and serial number.	Date of infection.	Febrile reaction, days.	Passage of brain to 2nd guinea-pigs, days after infection.	Result.	Confirmatory passage to 3rd guinea-pigs from spleen result.
Wooldridge .. 229	7–1–39	9–14	107	Negative.	Negative.
Dennison .. 221	16–1–39	11–15	98	,,	,,
Dyas .. 24	20–1–39	7–14	92	,,	,,
Dennison .. 246	24–4–39	7–14	66	,,	....
Dyas .. 52	8–5–39	8–14	63	Positive. F e v e r. Rickettsia +.	Died 7th day.
Dyas .. 69	10–7–39	11–14	49	D o u b t f u l. N o fever. Spleen +.	Negative.
Dyas .. 55	5–6–39	13–14	35	Positive. F e v e r. Ascites. Rickett-sia +.	Positive. Fever. Ascites.
Dyas .. 71	24–7–39	8–14	25	Positive. F e v e r. Rickettsia +.	Positive. Fever. Ascites.

Conclusion.—XK human strains of typhus have been recovered from passage guinea-pigs by injection of brain emulsions intraperitoneally to guinea-pigs at periods up to 63 days after infection but not later. This evidence suggests that the reservoir of XK typhus injection in nature may not be in susceptible animals but perhaps in arthropod vectors.

<p style="text-align:center">ARTIFICIAL INFECTION OF SIMLA HILLS WILD RATS, *Rattus rattus*, WITH HUMAN XK STRAINS OF TYPHUS.</p>

These wild rats and their fleas furnish a ready source of X19 murine typhus. Intraperitoneal infection with human XK strains was carried out, using spleen emulsions of passage guinea-pigs. The results were as follows :—

XK human strain Dennison—four wild rats infected 22nd May, 1939.

1. Died 7th day.
2. Killed 8th day. P.M. negative. Spleen passage to guinea-pig gave X19 reaction (fever 10th to 14th days, scrotal reaction 11th day, Rickettsia +).
3. Died 9th day.
4. Killed 14th day (moribund). P.M. marked emaciation. Spleen passage to guinea-pig negative.

XK human strain Wooldridge—four wild rats infected 22nd May, 1939.

1. Died 2nd day.
2. Killed 8th day. P.M. negative. Spleen passage to guinea-pig positive (fever 10th to 13th days, ascites, no scrotal reaction, Rickettsia +).
3. Killed 14th day (moribund). P.M. marked emaciation. Spleen passage to guinea-pig gave X19 reaction (fever 10th to 14th days, scrotal reaction 12th day, Rickettsia +).
4. Killed 28th day (moribund). P.M. marked emaciation. Spleen passage to guinea-pig gave X19 reaction (fever 10th to 14th days, scrotal reaction 10th day, Rickettsia not seen). Brain passage to guinea-pig gave a febrile reaction only, P.M. negative, Rickettsia not seen.

Conclusion.—After intraperitoneal injection with human XK typhus strains, six out of eight wild rats were dead or moribund after periods varying from two to 28 days. In three cases a typical X19 reaction was obtained on passage. In only one case out of eight could the XK strain be re-established on animal passage.

EXPERIMENTAL TYPHUS INFECTION IN WHITE RATS.

White rats of the Pasteur Institute strain were injected intraperitoneally with spleen emulsions of passage guinea-pigs showing typical evidence of infection with the X19 murine and XK human strains of typhus respectively. These rats showed no sign of illness and on post-mortem examination there was no scrotal reaction or ascites or splenic enlargement. Smears of scrapings from the tunica vaginalis, however, showed in all cases typical intracellular Rickettsia. Details are shown in Table III :—

TABLE III.

White rats inoculated with typhus.

Strain used.	Date of injection.	Days between injection and P. M.	Typical intracellular Rickettsia in smears from tunica vaginalis.
XK Wooldridge ..	16–2–39	9	+
XK Dyas ..	16–2–39	9	+
XK Dennison ..	27–2–39	7	+
XK Dyas ..	11–4–39	5	+
XK Dyas ..	11–4–39	10	+
XK Dyas ..	11–4–39	15	+
X19 murine ..	14–2–39	9	+
X19 murine ..	12–4–39	5	+
X19 murine ..	12–4–39	9	+

Conclusion.—The laboratory strain of male white rats when injected with either X19 murine or XK human strains show neither symptoms nor signs but only 'infection inapparente'. Rickettsia are definitely more readily demonstrable than in the case of guinea-pigs. Covell (1936*a*) noted this in the case of X19 strains.

NEGATIVE FINDINGS IN STRAY DOGS.

The blood serum of a number of pariah dogs from the plains and from the Simla Hills was tested against X19 and XK *Proteus* suspensions with the results shown in Table IV :—

TABLE IV.

Weil-Felix reaction in pariah dogs.

Dogs tested.	Number.	X19.		XK.		
		Negative.	+ 1 : 50	Negative.	+ 1 : 25	+ 1 : 50
From plains	87	86	1	46	27	14
From Simla Hills— February 1939 ..	7	7	..	2	3	2
July 1939	6	6	..	4	1	1

Eight stray dogs captured near Kasauli were also tested for typhus infection by intraperitoneal injection to male guinea-pigs of the washed blood clot. No evidence of typhus infection was obtained.

NEGATIVE FINDINGS WITH BED-BUGS.

Three batches of bed-bugs (the species of temperate climates *Cimex lectularius*) were fed on XK-infected guinea-pigs and one batch on an X19-infected guinea-pig. Subsequent feeding experiments and animal inoculation experiments produced no evidence that bed-bugs are capable of transmitting typhus or that they can even harbour the infection.

NOTE ON THE HUMAN XK STRAINS EXAMINED.

The XK strains Wooldridge, Dennison and Dyas were originally isolated from European patients by Dr. R. O. A. Smith and were received from him about the 68th, 68th, and 10th guinea-pig passages, respectively.

A strain has since been isolated from Gauri Shanker Atri, an adult male Indian villager, who complained of severe headache and backache with fever and sleeplessness of one week's duration. The blood was drawn into $1\frac{1}{2}$ per cent citrate at 7 a.m. but could not be made use of in the laboratory until 2 p.m. By this method it appears to be possible to isolate typhus strains later in the illness than had been supposed and even when there is several hours delay in infecting the experimental animals.

Fever cases are common among the villagers in the Simla Hills about the end of the monsoon and it has been supposed that these might be cases of typhus. The villagers do not encourage medical investigation and it is rarely possible to obtain even one sample of blood from a febrile patient. However, specimens were secured from 25 cases during August and September which, from the meagre histories obtainable, might possibly have been cases of typhus. Twenty-one of these were discarded at once as films of their blood showed heavy malarial infection. One further case gave a highly positive Widal reaction against *Paratyphosus* B. The remaining three cases were tested for typhus and one positive result was obtained, the above G. S. A. The Weil-Felix test on his blood serum gave X19 negative, XK + 1 : 50 only. For a week or two before his fever commenced he had noticed that he was being severely bitten while at work in the fields. He could not see the biting agent and larval mites are suspect.

NOTE ON THE RICKETTSIA.

In infections with both the X19 murine and the XK human strains the Rickettsia appear as miniature bacteria in the protoplasm of the endothelial cells of the tunica vaginalis of male guinea-pigs and white rats. 'Much patience is required in the examination of a smear of infected material in order to find them. Half an hour's search may prove fruitless' (Lewthwaite and Savoor, *loc. cit.*). Extracellular organisms have sometimes obviously escaped from a ruptured cell but generally speaking it is wiser to ignore suspicious organisms apart from those which are typical and intracellular. After three or four hours staining with 1 : 10 Giemsa the Rickettsia of the XK type are on the whole more deeply stained than those of the X19 type. The colour varies, being sometimes definitely blue but usually some shade of purple. The Rickettsia vary considerably in size and shape. Those of the X19 type are most often thin bacilliform- or diphtheroid-like organisms while those of the XK type are usually bi-polar staining bacilliform- or diplococcus-like organisms. The X19 type tend to be longer and thinner while in the XK type, the ends of the organisms are more definitely rounded. It is not always possible to distinguish the one from the other. Infected cells may show only a few scattered organisms or the protoplasm may be packed with a compact mass of Rickettsia so that the individuals cannot be distinguished. Clumps of Rickettsia are sometimes haloed, being surrounded by a lighter-stained area of protoplasm. Intranuclear Rickettsia are never seen and other types of cells are not infected. Smears of the peritoneal exudate or smears of scrapings from the peritoneum especially from the spleen surface may also show Rickettsia. Spleen smears occasionally show typical intracellular Rickettsia and frequently Rickettsia-like extra- or intracellular bodies.

In the Rocky Mountain Spotted Fever group of infections the appearances are entirely different (Pinkerton, 1936). There are generally less than 15 organisms to an infected cell and rarely more than 25. The individuals are larger and often definitely haloed. Macrophage cells are infected as well as serosal cells. In tissue culture the cell nuclei are also invaded by the Rickettsia.

Mention may be made of a rapid stain for Rickettsia described by Laigret and Auburton (1938). It is useful for diagnosis and especially as a preliminary in

the selection of specimens for more elaborate staining. For good differentiation nothing has been found better than the Giemsa-stained smears cleared in xylol as described by Covell (1936*b*).

LABORATORY METHODS IN TYPHUS WORK.

A summary of suitable methods may be useful to other workers in India. The male guinea-pig is the experimental animal of choice in typhus work. For the isolation of tsutsugamushi strains the Malayan workers resort to the intraocular infection of rabbits. Suspected material, e.g., human blood, brain emulsions from suspected animal reservoirs, spleen or brain emulsions of passage animals or emulsions of arthropods, should be injected intraperitoneally. The human blood may· be in the form of a saline suspension of the washed cells deposited in 1½ per cent citrate solution or a saline suspension of the washed clot after removal of serum (Giroud's method quoted by Lewthwaite and Savoor, *loc. cit.*). The tissues or arthropods for passage may be ground up in 10 c.c. saline, the emulsion filtered through muslin and 5 c.c. injected to each of two guinea-pigs. Secondary infections are common and the material should be handled in as sterile a manner as possible.

A febrile reaction in the guinea-pigs should be looked for. It may start by the fifth day or be delayed as late as the 14th day and it may last for five days or more. There is generally a longer incubation period after brain passage than after spleen passage. With the guinea-pigs used the normal rectal temperature was between 101·6°F. and 102·4°F. Anything over 102·8°F. was regarded as febrile and during the febrile period the temperature varied usually between 103°F. and 104·5°F. but temperatures as high as 105·7°F. were observed. If both passage animals die or fail to react the stock strains may still be recovered by brain passage of surviving guinea-pigs of earlier passages up to two months after infection.

In the case of X19 infections the scrotal reaction may be expected to show about the 5th to 8th days and it lasts for from three to seven days.

Post-mortem examination and passage are conveniently carried out when the scrotal reaction is passing off in the case of X19 infections or about the 14th day in the case of XK infections. In both types the spleen is usually enlarged and dark, and sometimes shows a whitish surface film. In XK infections there is often abundant clear viscid fluid in the peritoneal cavity. In one series 34 out of 45 passage animals showed ascites about the 14th day.

Smears of scrapings from the tunica vaginalis show typical intracellular Rickettsia in more than half the passage animals. In X19 infections the best time for the demonstration of Rickettsia is when the scrotal reaction first appears. Later on, the appearances are spoilt by the presence of masses of the granules described by Mooser (1928).

To summarize : Both types give a febrile reaction, both show enlarged spleen and Rickettsia can often be demonstrated in the tunica vaginalis : scrotal reaction is characteristic of the X19 murine type and ascites is a feature of the XK human type.

The white rat is useful if good specimens of Rickettsia are required—after intraperitoneal infection numerous Rickettsia-infected cells may be found in scrapings from the tunica vaginalis taken between, say, the 5th and 15th days.

The most useful animal for the demonstration of agglutinins is probably the rabbit. The blood serum examined at weekly intervals after intraperitoneal infection will sometimes, but by no means always, show a significant rise of agglutinins against *Proteus* X19 or XK suspensions respectively.

Summary.

1. The suspected vector of the XK type of typhus in the Simla Hills, the larval *Trombicula deliensis*, has been found as a natural ectoparasite of the brown monkey, *Silenus (Macacus) rhesus*. Attempts to isolate typhus strains from these monkeys and from larval mites removed from them have so far been unsuccessful. Intraperitoneal injection of these monkeys with XK human strains of typhus gave negative results. The evidence is not sufficient to eliminate the possibility that monkeys are concerned in the spread of typhus.

2. XK human strains of typhus have been recovered from passage guinea-pigs at periods up to 63 days but not later. The reservoir of XK typhus infection between one typhus season and the next may be in the (unknown) arthropod vector.

3. X19 murine strains of typhus can be recovered from wild rats in the Simla · Hills with great regularity. The isolation of XK strains from the same source (if they are actually present) is thereby rendered difficult, and attempts have so far been unsuccessful.

4. A laboratory strain of white rats showed no symptoms after infection with either X19 murine or XK human strains of typhus, but Rickettsia are readily demonstrable.

5. An XK human strain of typhus was isolated from an Indian patient about the end of the first week of the illness and when there had been a delay of several hours before animal inoculation was carried out.

6. Negative findings are recorded regarding the implication of (*a*) pariah dogs and (*b*) bed-bugs in typhus epidemiology.

7. Notes are given on the Rickettsia and on laboratory methods in typhus work.

8. The observations on Himalayan strains of typhus have given similar results to those reported from Malaya where much more extensive investigations were carried out (Lewthwaite and Savoor, *loc. cit.*).

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THE NICOTINIC-ACID CONTENT OF CEREALS AND PELLAGRA.

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THE remarkable effect of nicotinic acid in the treatment of pellagra has been conclusively demonstrated by Spies and his collaborators (1938), and a considerable number of other workers in various countries. The trial of nicotinic acid in the treatment of pellagra was prompted by the observation of Elvehjem *et al.* (1937) that nicotinic acid cures black-tongue in dogs, a condition which has for a number of years been considered analogous to human pellagra.

The association of pellagra with a maize diet has been recognized for more than a century. While occasional cases of pellagra may occur among populations consuming cereals other than maize, pellagra as a large scale public health problem exists only in countries in which maize is the staple food. An example of the latter is Roumania. In certain maize-eating districts in Roumania, 5 per cent or more of the population may show signs of pellagra at the season of greatest prevalence (Aykroyd, Alexa and Nitzulescu, 1935). We may contrast this high incidence with the rarity of classical pellagra in the rice-eating areas of India. A few cases of primary and secondary pellagra have been reported here and there, but the disease never assumes epidemic proportions. No theory of causation can be regarded as satisfactory which does not explain the maize-eater's liability to the disease.

Since nicotinic acid improves or cures pellagra, it is natural to suppose that the disease is directly due to nicotinic-acid deficiency in the same way that beri-beri is directly due to vitamin-B_1 deficiency. The development of the cyanogen-bromide method for estimating nicotinic acid in biological materials (Swaminathan, 1938, 1939), which has been employed with slight modifications by a number of other workers (Bandier, 1939; Bandier and Hald, 1939; Pearson, 1939), made it possible to test this hypothesis. In the present investigation the nicotinic-acid content of various cereals, including maize, has been determined and the nicotinic-acid content of a maize diet associated with pellagra has been compared with that of a typical poor rice diet.

EXPERIMENTAL.

The method employed has been described in detail before (Swaminathan, *loc. cit.*). The following is a brief summary :—

> A weighed amount (20 g. to 30 g.) of the finely-ground test material is extracted with hot water. Protein and its derivatives are removed with lead acetate and the excess of lead as lead sulphate. The extract is evaporated to a small bulk (about 100 ml.). Concentrated hydrochloric acid is added to make about a 5 per cent solution, and the solution boiled for 30 minutes to convert nicotinamide into the acid. The solution is decolorized with charcoal in an alkaline medium (pH 10), brought to pH 7, and made up to volume (150 ml. to 200 ml.).
>
> The nicotinic acid present was estimated in aliquots (5 ml. to 10 ml.) of the extract, after treating with the cyanogen-bromide-aniline reagent, in the presence of 2 ml. of phosphate buffer. The resulting colours were compared against those obtained with standard nicotinic acid (10 μg. to 20 μg.) treated in the same manner.

The results of determinations carried out on various cereals and cereal products are shown in Table I :—

TABLE I.

Nicotinic-acid content of various cereals and cereal products.

Mg./100 g.

Maize, whole, white (mean of 9 Indian samples).	1·3	
Maize, whole, yellow (mean of 9 Indian samples).	1·4	
Maize, cornflour, refined	0·3	
Rice, raw, home-pounded (mean of 4 varieties).	2·4	
Rice, raw, milled (mean of 9 varieties).	1·6	
Rice, parboiled, home-pounded (mean of 4 varieties).	4·0	
Rice, parboiled, milled (mean of 9 varieties).	3·8	
Rice polishings	8·8	
Wheat, whole (sample 1)	5·3	
Wheat, „ („ 2)	4·7	
Wheat, refined, white, flour (sample 1)	1·1	
Wheat, „ „ „ („ 2)	0·9	
Wheat germ	9·1	
Barley (sample 1)	2·5	
Barley („ 2)	3·0	
Oats, 'medium' (sample 1)	1·0	
Oats, 'medium' („ 2)	1·1	
Millets :—		
Cambu (*Pennisetum typhoideum*) (sample 1)	2·1	
Cambu „ „ („ 2)	2·0	
Cholam (*Sorghum vulgare*) (sample 1)	1·4	
Cholam „ „ („ 2)	1·5	
Ragi (*Eleusine coracana*) (mean of 5 samples)	1·4	

Of the products tested, whole wheat stands the highest. Home-pounded raw and parboiled rice, milled parboiled rice, and barley, come next in order. There is little to choose between whole maize, milled raw rice, and the various millets. The lowest figures were given by oats, de-germinated maize meal, and refined wheat flour. No light was thrown on the association between maize and pellagra by this

series of tests. The whole maize samples were low in nicotinic acid but not very different in this respect from various other cereal products.

The maize tested was all grown in India. We felt it possible that Indian maize might differ in nicotinic-acid content from maize grown elsewhere in the world, or that maize actually consumed by pellagrins might have a particularly low value. Accordingly, we obtained samples of maize from parts of the United States and Roumania where pellagra is known to occur. Two samples were supplied by Dr. T. D. Spies, Cincinnati, Ohio; one of these was whole meal, the other a highly-milled de-germinated product, both obtained from white maize. Some 25 specimens were sent by Dr. J. Nitzulescu, Jassy, Roumania. These were whole, yellow, unground maize and obtained directly from peasant families living in the pellagra district of Moldavia and growing maize for their own use. Some of the families concerned contained cases of pellagra. The nicotinic acid of various samples of maize from U. S. A. and Roumania is shown in Table II :—

TABLE II.

The nicotinic-acid content of maize samples obtained from places in U. S. A. and Roumania where pellagra is known to occur.

Mg./100 g.

		REMARKS.
Maize meal, whole, white, U. S. A. ..	.. 1·6	
,, ,, de-germinated, white, U. S. A.	.. 0·9	
Maize, whole, yellow, Roumania ..	.. 1·2	From a family with 3 pellagra cases.
,, ,, ,, ,, ..	.. 1·3	,, ,, ,, 1 ,, case.
,, ,, ,, ,, ..	.. 1·3	,, ,, ,, 1 ,, ,,
,, ,, ,, ,, ..	.. 1·4	,, ,, ,, 1 ,, ,,
,, ,, ,, ,, ..	.. 1·3	,, ,, ,, 1 ,, ,,
,, ,, ,, ,, ..	.. 1·5	,, ,, ,, 4 ,, cases.
,, ,, ,, ,, ..	.. 1·6	No pellagra in family.
,, ,, ,, ,, ..	.. 1·4	,, ,, ,,
,, ,, ,, ,, ..	.. 1·4	,, ,, ,,
,, ,, ,, ,, ..	.. 1·6	,, ,, ,,
Mean of samples from families with pellagra	.. 1·3	
,, ,, ,, without ,,	.. 1·5	
Mean of Indian samples (yellow) ..	.. 1·4	

The values given by the American and Roumanian samples were similar to those given by the Indian samples. There was little difference between the samples

obtained from families with or without pellagra, which suggests that variations in the nicotinic-acid content of maize play no part in the ætiology of the disease. Of the American samples, the whole maize-meal product gave a considerably higher figure than the de-germinated product. This is a point of some interest, since certain American workers have maintained that the outbreak of pellagra in U. S. A. in the early years of the present century was due to the widespread introduction of the de-germinated maize meal. It is to be observed, however, that pellagra occurs in severe form in Roumania among peasants consuming whole, yellow, maize.

The nicotinic-acid content of rice.—Table III shows the details of the analyses of rice samples summarized in Table I:—

TABLE III.

Nicotinic-acid content of varieties of rice.

Mg./100 g.

Variety.	Raw.		Parboiled.	
	Home-pounded.	Milled.	Home-pounded.	Milled.
Vadan Samba	..	1·5	..	3·5
CO 9	..	1·5	..	3·1
ADT 2	..	1·6	..	3·6
ADT 11	2·1	1·7	4·1	3·6
GEB 24	..	2·3	..	4·8
CO 7 (1st sample) ..	..	1·8	..	4·7
CO 7 (2nd sample) ..	2·2	1·1	3·8	3·4
CO 5	2·8	1·3	4·6	4·1
CO 2	2·4	1·7	3·3	3·0
Average ..	2·4	1·6	4·0	3·8

A striking finding was the relatively high nicotinic-acid content of milled parboiled rice. In the parboiling process, rice is soaked in the husk for 24 to 36 hours, subjected to steam at atmospheric pressure for 15 to 20 minutes and subsequently dried in the sun. It was shown by Aykroyd (1932) that during the process of parboiling some of the vitamin B_1 present in the germ and pericarp diffuses through the grain and cannot be removed on subsequent milling. The results shown in Table III suggested that nicotinic acid behaves in the same way.

To investigate this point further, samples of raw and parboiled rice milled to specific degrees were obtained. A hand machine for milling rice samples, similar to that used in the earlier experiments (Aykroyd, 1932) was kindly lent by the Biochemical Department, Indian Institute of Science, Bangalore. The loss of weight occurring in samples of rice subjected to milling was used as the criterion of the degree of milling. The machine mills rice cleanly with few breakages and the loss of weight is almost entirely due to the removal of the outer layers.

Two varieties of rice were used, CO 9, a large-grained red rice, and ADT 2 a small-grained white rice. These varieties, in the raw and parboiled state, were

milled to various degrees and the nicotinic-acid content of the resulting samples determined. The values obtained are shown graphically in Charts 1 and 2. The

CHART 1.
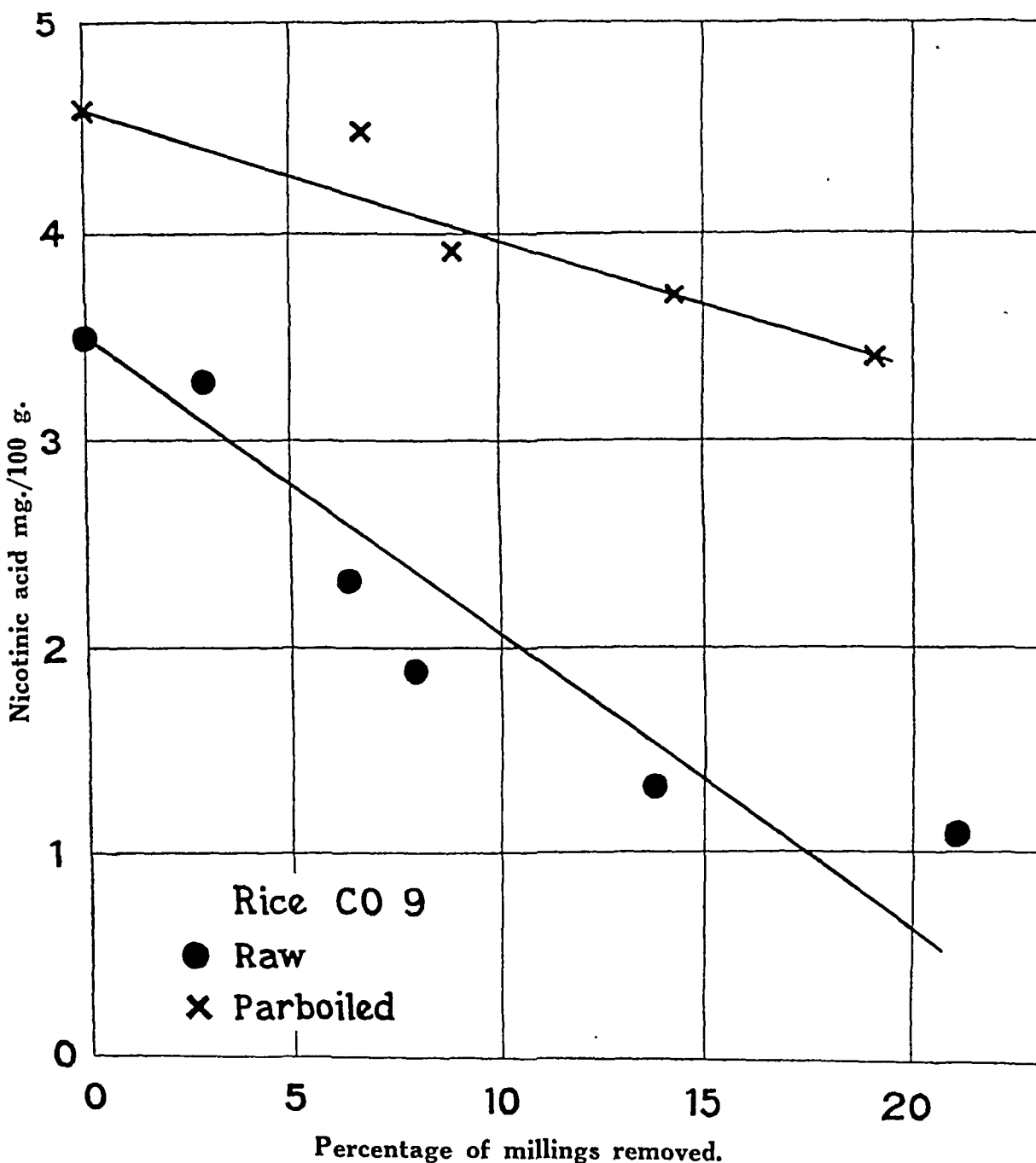
The effect of milling in the raw and parboiled state on nicotinic-acid content. (Rice CO 9).

nicotinic-acid content of husked rice, i.e., rice in which the germ and pericarp is almost intact, is 3 mg. to 5 mg. per cent. Raw rice when pounded loses about half the nicotinic acid originally present and when machine-milled about two-thirds.

CHART 2.
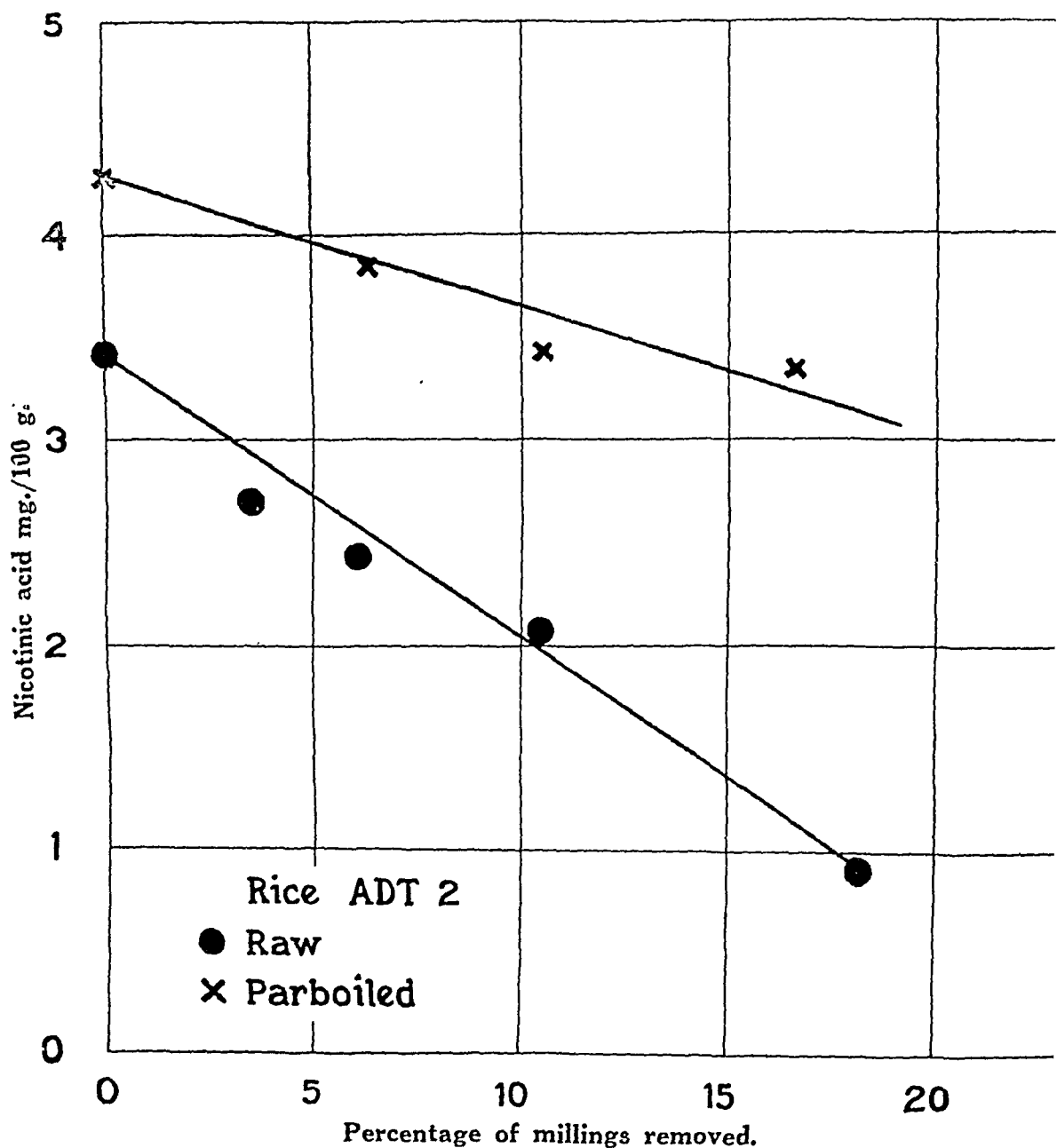
The effect of milling in the raw and parboiled state on nicotinic-acid content. (Rice ADT 2).

This shows that most of the nicotinic acid in the rice grain is concentrated in the germ and pericarp.

The charts demonstrate very clearly the effect of parboiling. The parboiled samples, even when very highly milled, retained more than 3 mg. of nicotinic acid per cent. While milled parboiled rice is superior to whole maize in nicotinic-acid content the values given by milled raw rice and maize are of the same order.

Washing and cooking.—In preparation for consumption, rice is usually washed 3 times. The amount of water used in the subsequent cooking varies according to custom. Some communities or groups consume the cooking water; others discard it. In the experiments summarized in Table IV, the rice was washed 3 times and cooked in rather more than enough water to cover it :—

TABLE IV.

The effect of washing and cooking on the nicotinic-acid content of rice.

Serial number.	Rice variety.	Method of preparation.	Uncooked rice, mg./100 g.	Cooked rice, mg./100 g.	Percentage remaining in cooked rice.	Percentage lost in wash water.	Percentage lost in conjee.
1	CO 2	Raw, hand-pounded ..	2·4	0·6	25	50	25
2	,,	Parboiled, hand-pounded ..	3·3	1·3	40	10	40
3	,,	Raw, machine-milled ..	1·7	0·6	30	55	15
4	,,	Parboiled, machine-milled ..	3·0	1·3	40	10	50
5	CO 5	Raw, hand-pounded ..	2·8	1·3	45	..	..
6	,,	Parboiled, hand-pounded ..	4·6	1·9	40	..	..
7	,,	Raw, machine-milled ..	1·3	0·5	40	..	..
8	,,	Parboiled, machine-milled ..	4·1	1·8	45	..	..
9	CO 7	Raw, hand-pounded ..	2·2	0·9	40	..	..
10	,,	Parboiled, hand-pounded ..	3·8	1·7	45	..	..
11	,,	Raw, machine-milled ..	1·1	0·4	35	..	..
12	,,	Parboiled, machine-milled ..	3·4	1·4	40	..	..
		AVERAGE ..	..	..	40	..	..

Washing and cooking in this manner were found to reduce the nicotinic-acid content of rice by 50 to 60 per cent. Allowing for differences in custom, we may reckon that on the average rice loses 40 to 50 per cent of its nicotinic acid during domestic preparation. It is important to note that the values for raw milled rice when cooked are very considerably below those given by any sample of maize.

The nicotinic-acid content of maize and rice diets.—The frequency of pellagra among poor maize-eating populations, and its rarity among poor rice-eating populations, cannot then be explained in terms of the nicotinic-acid content of these cereals, as determined by the cyanogen-bromide test. There remains the possibility that the poor rice-eater obtains greater quantities of nicotinic acid from ingredients in the diet other than his staple cereal. Data are available for the investigation of this question. In India some 15 diet surveys have been carried out on rice-eating groups in widely-separated areas of the country. These have revealed that the diet of the poor rice-eater is astonishingly uniform throughout India. The rice diet shown in Table V, based on the results of surveys, is typical, and such as is consumed by millions in India.

For information about poor maize diets we can turn to a survey made in a pellagrous district in Moldavia (Aykroyd, Alexa and Nitzulescu, *loc. cit.*). This investigation covered 22 families during the winter and spring of 1933-1934. Of the 22 families, 17 contained members who had suffered from pellagra in the spring of 1933. A picture of the diet consumed by poor Roumanian families liable to suffer from pellagra was provided by the survey.

Table V compares the nicotinic-acid content of this diet with a typical poor rice-eater's diet. The nicotinic-acid values are based on determinations carried out on the uncooked foodstuffs. Maize was given a value of 1·4 mg. per cent. The nicotinic-acid content of the rice diet has been calculated on four assumptions, namely, that the main ingredient is (*a*) uncooked raw milled rice, (*b*) cooked raw milled rice, (*c*) uncooked parboiled milled rice, and (*d*) cooked parboiled milled rice. Since rice must always be cooked, (*a*) and (*c*) have no reference to practice and are inserted for purposes of comparison. Both raw and parboiled rice are consumed in India. No information is available as to losses in the nicotinic-acid content of maize on cooking. These must be very much smaller than those occurring in the case of rice, since maize is not washed before cooking, and in such districts as Moldavia is made into a species of pudding or cake ('mammaliga') with the addition of only small quantities of water for cooking.

Apart from the staple cereal, the diets bear a fairly close resemblance. They contain 40 g. to 80 g. of pulses, 50 g. to 100 g. of fruit, little or no milk, and small or negligible quantities of meat. The Roumanian diet has a slight advantage as regards the abundance of non-cereal ingredients. In both diets the cereal supplies the greater part of the nicotinic acid. The calorie yield of the Roumanian diet is considerably in excess of that of the Indian diet.

The nicotinic-acid content of these maize and rice diets, as estimated by the cyanogen-bromide test, thus fails to explain why one diet is pellagra-producing, the other not. The rice-eater, if he consumes his rice in the raw milled state, obtains less nicotinic acid than the poor Roumanian maize-eater who suffers from pellagra.

TABLE V.

The nicotinic-acid content of maize and rice diets.

Maize diet (Roumania).			Rice diet (India).		
Ingredients.	Grammes per adult male. (Consumption unit.)	Nicotinic acid (mg.).	Ingredients.	Grammes per adult male. (Consumption unit.)	Nicotinic acid (mg.).
Whole maize	762·0	10·67	Raw milled rice, uncooked ..	596·0	9·54
Whole wheat	2·0	0·10	Raw milled rice, cooked ..	596·0	3·00
Refined wheat flour ..	40·0	0·40	Parboiled milled rice, uncooked	596·0	22·65
Bread, brown	43·0	0·40	Parboiled milled rice, cooked ..	596·0	8·94
Bread, white	9·0	0·04	Dhal arhar	20·0	0·90
Rice	16·0	0·32	Black gram (*Phaseolus mungo*)	20·0	0·50
Milk	11·0	0·16	Brinjal (*Solanum melongena*) ..	28·0	0·28
Butter milk and skimmed milk.	5·1	0·08	Amaranth (*Amaranthus gangeticus*).	14·0	0·14
Eggs	0·5	0·01	Raw plantain	14·0	0·14
Meat	34·0	1·79	Meat	2·0	0·10
Beans	86·0	0·86	Gingelly oil	3·0	..
Other vegetables and fruits	100·0	0·60	Coco-nut	2·0	0·05
TOTAL ..	..	15	TOTAL :—With raw milled rice, uncooked.	..	12
			With raw milled rice, cooked.	..	5
			With parboiled milled rice, uncooked.	..	25
			With parboiled milled rice, cooked.	..	11

DISCUSSION.

In another paper published from the Laboratories (Aykroyd, Krishnan and Passmore, 1939), evidence has been brought forward that pathological conditions

due to nicotinic-acid deficiency may occur in individuals consuming rice diets. Stomatitis resembling that observed in pellagra, often associated with other lesions of muco-cutaneous junctions, is very common among poor rice-eaters. Symmetrical dermatitis, diarrhœa, and mental changes, which are cardinal signs of pellagra, are not observed in these cases. Large doses of nicotinic acid produced improvement in a proportion of stomatitis cases, the improvement in the glossitis being particularly marked. It was observed that the excretion of nicotinic acid in these subjects, both before and after receiving a test dose of nicotinic acid, was lower than that observed in normal individuals (Swaminathan, 1939).

We are not at present in a position to explain why the poor rice-eater, who may apparently suffer from nicotinic-acid deficiency, so rarely develops true pellagra. Presumably the missing link will be found in some difference between maize and rice other than nicotinic-acid content. When pellagra was generally supposed to be due to vitamin-B$_2$ deficiency, it was shown by Aykroyd (1930) that neither rice nor millet had any superiority over maize as a source of vitamin B$_2$ and it was concluded that ' vitamin-B$_2$ deficiency cannot be accepted as the *sole* cause of human pellagra, as this disease is almost invariably associated with the consumption of maize '. There is a certain parallelism between the earlier investigation and the present one.

SUMMARY.

1. The nicotinic acid of a number of cereals and cereal products has been determined by the cyanogen-bromide method. Whole wheat was found to stand the highest. Home-pounded raw and parboiled rice, milled parboiled rice, and barley came next in order. Maize, milled raw rice, and the various millets gave low value of a similar order.

2. Maize obtained from parts of the United States and Roumania, where pellagra is known to occur, was found to have a nicotinic-acid content similar to that of Indian maize. Maize obtained from Roumanian families containing cases of pellagra did not give lower values than other samples.

3. The nicotinic acid present in rice is concentrated in the germ and pericarp. When rice is parboiled, nicotinic acid diffuses through the grain and cannot be removed on milling. Washing and cooking may remove some 50 per cent of the nicotinic acid present in rice.

4. The nicotinic-acid content of a poor maize diet associated with pellagra and of a typical poor rice diet was compared. The latter was estimated to contain less nicotinic acid than the former. Comparison of the nicotinic-acid content of poor rice and maize diets, as determined by the cyanogen-bromide method, thus fails to explain the association of pellagra and maize.

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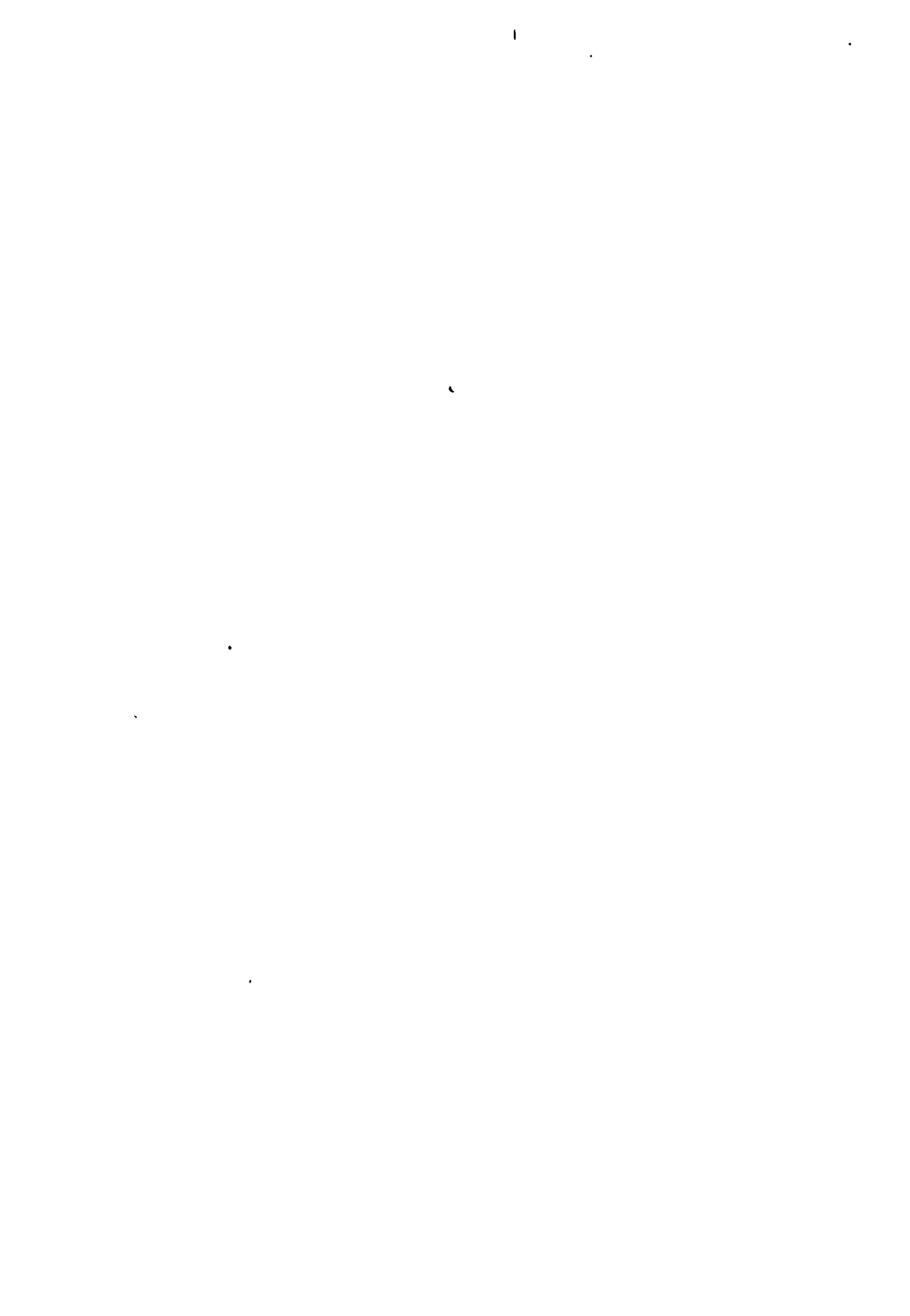
THE SYNTHESIS OF NICOTINIC ACID BY RATS.

BY

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A NUMBER of workers (Rhoads and Miller, 1935 ; Birch *et al.*, 1935, 1937 ; Harris, 1937) have shown that rats can survive and thrive fairly well on diets which may produce pellagra in human beings, black-tongue in dogs, and deficiency states in pigs and monkeys. It seems to follow that rats do not need, or can synthesize, the pellagra-preventive factor, now identified with nicotinic acid (Elvehjem *et al.*, 1937). Macrae and Edgar (1937), Cook *et al.* (1937), and Dann and Subbarow (1938), were unable to show that nicotinic acid and its derivatives are dietary essentials for rats. Chick *et al.* (1938) noted no increase in growth when rats fed on a maize diet received a nicotinic-acid supplement. On the other hand, certain workers (Frost and Elvehjem, 1937 ; Euler *et al.*, 1936, 1938 ; Helmer and Fouts, 1938) have reported that nicotinic acid and its derivatives may, under certain conditions, have an effect on the growth rate of rats.

The purpose of the present investigation was to find out whether rats require nicotinic acid for maintenance and growth or whether they can synthesize it.

EXPERIMENTAL.

Four groups of young rats (12 in each), with an average weight of 60 g., were fed respectively as follows :—

Groups I and II received diet 1 and groups III and IV were given diet 2 (Table I). Groups II and IV received in addition 1·25 mg. of nicotinic acid per day per rat mixed with the diet.

The diets were thoroughly mixed, and made into a paste by adding distilled water in order to prevent scattering of food. The weights of the diets before and after the addition of water were noted. In weighing out the daily food, care was taken that the food given should not exceed greatly the average food consumption, thus avoiding a large residue. Food remaining uneaten was collected daily,

dried in an air oven at 100°C., and the corresponding dry weights of the residual food subtracted from the dry weight of food given, to obtain the food intake.

TABLE I.

Composition of diets.

Ingredients.	Diet 1 (parts).	Diet 2 (parts).
Casein ..	25	25
Starch ..	59	47
Maize (white) ..	..	12
Coco-nut oil ..	10	10
Cod-liver oil ..	3	3
Salt mixture ..	3	3

In addition to the diet each rat received daily 10 μg. of crystalline vitamin B_1 and 40 μg. of flavine.*

The test diets were given for 9 weeks and 3 days, the first 3 days being regarded as a preparatory period allowing the rats to accommodate themselves to the diets. The rats were weighed weekly.

Four rats from each group were kept individually in metabolism cages. The urines and fæces of these were collected daily. To prevent decomposition of urine, 10 c.c. of 5 per cent HCl were placed in the flasks used for collection of urine. The metabolism cages, funnels, separators, and flasks placed for the collection of urine were washed down daily with small quantities of distilled water and the washings added to the respective urine bottles. The urine and washings were daily filtered through cotton-wool. The fæces were transferred daily into a small wide-mouthed bottle and dried in an air oven. Urine and fæces were analysed for nicotinic acid as described below :—

The analysis of urines, fæces, and tissues, for nicotinic acid.—Urine and washings collected over a period of one week were concentrated to a small volume (about 100 ml.) and the procedure previously described (Swaminathan, 1939) for human urine was applied. Weekly collections of fæces were dried and ground to a fine powder, and nicotinic acid estimated, the procedure adopted being the same as for foodstuffs and tissues (Swaminathan, 1938).

Muscle and liver.—The analytical procedure adopted was the same as previously described (Swaminathan, 1938) for liver.

*The flavine used in these experiments was kindly supplied by the Glaxo Laboratories, Ltd., Greenford, Middlesex, England.

Blood.—Blood filtrates obtained after the precipitation of proteins with tungstic acid were concentrated to a small bulk (about 10 ml. to 15 ml.). Concentrated hydrochloric acid, 2 ml. to 3 ml., was then added, and the mixture boiled for half an hour. Excess of $Ba(OH)_2$ was added to precipitate the sulphuric and tungstic acids present. The mixture was filtered and the barium in the filtrate removed as sulphate. The final solution was adjusted to pH 7 and made up to a convenient volume so that 1 ml. contained about 1 μg. to 1·5 μg. of nicotinic acid. The solutions in most cases had a light yellow colour which was allowed for by carrying out blank estimations.

The colorimetric procedure was essentially the same as that previously described (Swaminathan, 1939) for estimating the nicotinic-acid content of human urine.

RESULTS.

Intake and excretion of nicotinic acid.—Table II shows the intake and excretion of nicotinic acid by rats on different diets. It will be seen that the rats in groups I and III, receiving 5 μg. and 22 μg. daily, excreted on the average 36 μg. and 16 μg., and 40 μg. and 23 μg., in the urine and fæces, respectively. The quantities excreted per rat were thus 47 μg. and 41 μg. more than intake in the respective groups.

TABLE II.

Average intake and excretion of nicotinic acid.

Micrograms per rat per day.

Group number.	Intake.	EXCRETION.		Nicotinic-acid balance.
		Urine.	Fæces.	
I. Basal diet	5	36	16	− 47
II. Basal diet + nicotinic acid	1,135	60	23	+ 1052
III. Basal diet + maize	22	40	23	− 41
IV. Basal diet + maize + nicotinic acid.	1,212	60	28	+ 1124

Nicotinic-acid content of rat tissues.—It will be seen from Table III that there was no appreciable difference in the nicotinic-acid content of the liver, muscle, and blood, of the rats fed on the different experimental diets. The figures obtained in all the groups, including the groups in which intake of nicotinic acid was small, were close to those given by young rats of the same age fed on the Coonoor stock diet. This observation, taken in conjunction with the fact that excretion exceeded intake in groups I and III, proves that rats are able to synthesize nicotinic acid. The total amount of nicotinic acid excreted during 9 weeks was greatly in excess

of intake and the results shown in Table III indicate that the excess could not have been derived from the tissues.

TABLE III.

Nicotinic-acid content of different organs.

Group number.	Average body-weight, g.	Liver, mg. per 100 g.	Muscle, mg. per 100 g.	Blood, mg. per 100 c.c.
Initial values given by controls on stock diet.	62	15·2	4·6	0·94
I	107	15·0	2·0	0·95
II	109	17·1	3·3	0·95
III	136	13·8	3·2	0·84
IV	135	14·0	3·2	0·91

The nicotinic-acid excretion per rat in groups II and IV, receiving 1,135 μg. and 1,212 μg. daily, was only 60 μg. and 23 μg., and 60 μg. and 28 μg., in urine and fæces, respectively. The fate of the large excess of nicotinic acid not excreted is a problem for further investigation.

The addition of 1·25 mg. nicotinic acid to the basal diet, and the basal diet plus maize, did not produce greater gains in body-weight than these diets without the nicotinic-acid supplement (Table IV). These results are not in agreement with those of Frost and Elvehjem (1939). The introduction of 12 per cent of maize in the basal diet in place of an equivalent amount of starch definitely increased the rate of growth, which was presumably due to the fact that maize supplied factors in the vitamin-B_2 complex other than nicotinic acid and flavine.

TABLE IV.

Increase in body-weight of the rats on the different diets.

Group number.	Average initial body-weight, g.	Average final body-weight, g.	Gain in body-weight during 9 weeks, g.	Average weekly increase in body-weight, g.
I. Basal diet ..	61	107	46	5·1
II. Basal diet + nicotinic acid.	63	100	37	4·1
III. Basal diet + maize	63	136	73	8·1
IV. Basal diet + maize + nicotinic acid.	62	135	73	8·1

The question whether nicotinic acid is synthesized by the tissues of the rat or by intestinal bacteria, requires investigation.

SUMMARY.

1. Balance experiments were carried out for 9 weeks on 4 groups of rats. Two groups were given diets supplying about 5 μg. and 22 μg. of nicotinic acid daily, respectively. The other two groups received the same diets supplemented with about 1 mg. of nicotinic acid per rat per day. In the 2 groups fed on the unsupplemented diets the excretion of nicotinic acid per rat per day was 41 μg. and 47 μg., respectively, in excess of intake.

2. The groups of rats receiving about 1 mg. of nicotinic acid daily per rat excreted only 83 μg. and 88 μg. daily, respectively.

3. There was no appreciable difference between the nicotinic-acid content of the liver, muscles, and blood of the rats fed on the diets with and without additional nicotinic acid.

4. It is concluded that the rat can synthesize nicotinic acid.

5. The addition of nicotinic acid to the basal diet containing vitamin B_1 and flavine did not increase the growth-rate.

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THE PROTECTIVE ACTION OF TISSUES OF SCORBUTIC AND NORMAL GUINEA-PIGS AGAINST THE OXIDATION OF VITAMIN C AND THE NATURE OF THE PROTECTIVE MECHANISM.

BY

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ALTHOUGH vitamin C is rapidly oxidized *in vitro*, it is relatively stable in animal tissues. Metallic catalysts like $Cu^{\cdot\cdot}$ are present in tissues and body-fluids in such concentrations that without a protective mechanism the vitamin could never exist in them. It was first suggested by Green (1933) that living tissues possess some anti-oxidative mechanism. Later, de Caro (1934) and de Caro and Giani (1934) showed that most animal tissues contain a protective mechanism against the oxidation of vitamin C. The nature of the protective mechanism is, however, little understood. De Caro and Giani (*loc. cit.*) suggested that glutathione was the substance which caused stabilization of ascorbic acid in tissues. According to Mawson (1935), the protective action exerted by rat-tissue extracts could not wholly be explained by the presence of glutathione, cysteine, cystine, or hydrogen sulphide. He found it impossible to decide upon the nature of the protective mechanism, but his results show that in the fresh extract the protective action is due to several substances, some of which can be removed by dialysis and some precipitated by mercuric acetate or trichloracetic acid or by boiling. But it was found that the protective mechanism was not altered much by such treatment. Later, Giri (1938, 1939a) established a relationship between the catalytic oxidation of vitamin C and the activity of phosphatases. It was shown that the activity of tissue phosphatases was not influenced by vitamin-C-$Cu^{\cdot\cdot}$ complex in the presence of protective substances, while the activity was inhibited by the complex in their absence.

Recently, Courtois and Manouvrier (1939*a*, *b*) confirmed the findings of Giri that the activity of phosphatases is inhibited by vitamin C when the latter is oxidized by Cu$\cdot\cdot$, and this effect was noticed in the hydrolysis of various phosphoric esters. If, on the other hand, the oxidation of vitamin C is prevented by some protective mechanism, the activity of the enzyme is not affected by the vitamin-C-Cu$\cdot\cdot$ complex. Thus, it is clear that the protective mechanism possessed by animal tissues has important physiological functions.

The present paper describes investigations on the protective action of tissues of scorbutic and normal guinea-pigs against the catalytic oxidation of vitamin C by Cu$\cdot\cdot$ and the nature of the protective mechanism.

EXPERIMENTAL.

Guinea-pigs were fed on a scurvy-producing diet B already described (Giri, 1939*b*), and on the same diet supplemented with 5 mg. of ascorbic acid per day. The first group developed scurvy, while animals on the supplemented diet remained free from the disease.

Preparation of tissue extracts.—The guinea-pigs were drowned after 3 to 5 weeks on the diets; the liver, kidney, adrenal glands, and brain, were removed immediately after death and weighed. All the animals on the vitamin-C deficient diet had post-mortem signs of scurvy. The tissues were ground in a mortar with glass-powder, and extracted with 10 times the volume of phosphate buffer (M/15 pH 7·2 to 7·3) and centrifuged, after being kept at room temperature for 1 to 2 hours. The clear extract thus obtained after centrifuging was used for the study of protective action.

The protective action of the tissue extracts against the catalytic oxidation of vitamin C by Cu$\cdot\cdot$, was determined by estimating the rate of oxidation of ascorbic acid (B.D.H.) in presence and absence of the tissue extracts. The reaction was carried out at 37°C. and at pH 6·8 to 7·1. The composition of the reaction mixture was as follows :—

> 20 ml. M/15 phosphate buffer.
> 5 ml. of vitamin-C solution containing 5 mg. vitamin C.
> 1 ml. of $CuSO_4 5H_2O$ solution containing 0·063 mg. Cu$\cdot\cdot$.
> x ml. tissue extract.

The total volume of the reaction mixture was adjusted to 50 ml. Aliquots were removed at known intervals of time, and immediately added to an equal volume of 10 per cent trichloracetic acid, and titrated with 2 : 6-dichloro-phenol-indophenol dye, which was standardized against synthetic ascorbic acid.

The degree of protection exerted by the tissue extracts against the catalytic oxidation of vitamin C by Cu$\cdot\cdot$, is expressed as follows :—

$$\text{Degree of protection} = 100 \times \left\{ 1 - \frac{\text{The amount of vitamin C destroyed in the presence of the extract.}}{\text{The amount of vitamin C destroyed in the absence of the extract.}} \right\}$$

The experimental conditions are : 1 hour incubation at 37°C.; the volume of tissue extract used = 1 ml.; and the amount of added Cu$\cdot\cdot$ = 0·063 mg.

A. The protective action of tissue extracts of scorbutic and normal guinea-pigs.

The results of the experiments on the rate of oxidation of vitamin C by $Cu^{\cdot\cdot}$ in the presence and absence of tissue extracts are shown in Table I and the degree of protection exerted by the tissue extracts is indicated in Table II :—

TABLE I.

Protective action of tissue extracts against the catalytic oxidation of vitamin C by $Cu^{\cdot\cdot}$.

Guinea-pig number.	Reaction mixture.	LIVER.			KIDNEY.			ADRENALS.			BRAIN.		
		Mg. vitamin C in the total volume of the reaction mixture.											
	Time in minutes :—	0	30	60	0	30	60	0	30	60	0	30	60

A. Normal guinea-pigs.

Guinea-pig number.	Reaction mixture.	LIVER 0	30	60	KIDNEY 0	30	60	ADRENALS 0	30	60	BRAIN 0	30	60
1 (a)	Vitamin C + Cu··	5·0	0·6	0·2	5·0	0·6	0·2	5·0	0·6	0·2	5·0	0·6	0·2
(b)	Vitamin C + Cu·· + 1 c.c. extract	5·0	2·9	2·6	5·0	3·0	2·1	5·0	4·2	3·8	5·0	2·2	0·6
(c)	Vitamin C + Cu·· + 2 c.c. extract	5·0	4·2	3·4	5·0	3·8	3·4	..	..	..	5·0	2·4	1·2
2 (a)	,, ,, ,,	5·0	0·6	0·2	5·0	0·6	0·2	5·0	0·6	0·2	5·0	0·6	0·2
(b)	,, ,, ,,	5·0	3·0	2·2	5·0	2·5	1·5	5·0	4·0	1·8	5·0	2·0	0·6
(c)	,, ,, ,,	5·0	4·2	3·6	5·0	3·6	2·9	..	..	..	5·0	2·6	1·4
3 (a)	,, ,, ,,	5·0	0·7	0·2	5·0	0·4	0·2	5·0	1·0	0·2	5·0	0·7	0·2
(b)	,, ,, ,,	5·0	3·6	3·1	5·0	3·6	3·0	5·0	3·8	3·4	5·0	2·0	0·5
(c)	,, ,, ,,	5·0	4·4	3·8	5·0	4·4	4·0	..	..	..	5·0	2·4	1·2

TABLE I—*concld.*

Guinea-pig number.	Reaction mixture.	LIVER.			KIDNEY.			ADRENALS.			BRAIN.		
		Mg. vitamin C in the total volume of the reaction mixture.											
	Time in minutes :— ..	0	30	60	0	30	60	0	30	60	0	30	60
A. Normal guinea-pigs—concld.													
4 (a)	Vitamin C + Cu¨ + 2 c.c. extract ..	5·0	0·6	0·2	5·0	0·6	0·2	5·0	0·6	0·2	5·0	0·8	0·2
(b)	„ „ „ ..	5·0	3·0	2·0	5·0	2·9	1·7	5·0	4·2	4·0	5·0	2·0	1·0
(c)	„ „ „ ..	5·0	4·2	3·6	5·0	3·8	3·3	..	..	..	5·0	2·6	1·4
B. Scorbutic guinea-pigs.													
1 (a)	„ „ „ ..	5·0	0·3	0·2	5·0	0·6	0·2	5·0	0·4	0·3	5·0	0·4	0·3
(b)	„ „ „ ..	5·0	4·1	3·5	5·0	3·8	1·6	5·0	2·6	1·6	5·0	2·0	1·0
(c)	„ „ „ ..	5·0	4·8	4·4	5·0	5·0	4·4	..	..	..	5·0	3·4	2·4
2 (a)	„ „ „ ..	5·0	0·4	0·2	5·0	0·6	0·2	5·0	0·6	0·2	5·0	..	0·6
(b)	„ „ „ ..	5·0	4·4	4·2	5·0	3·2	2·2	5·0	3·6	3·0	5·0	1·8	0·6
(c)	„ „ „ ..	5·0	5·0	4·8	5·0	5·0	3·8	..	..	..	5·0	2·2	1·0
3 (a)	„ „ „ ..	5·0	0·6	0·6	5·0	0·6	0·4	5·0	0·6	0·2	5·0	0·6	0·4
(b)	„ „ „ ..	5·0	4·4	3·8	5·0	3·4	2·5	5·0	4·4	4·0	5·0	1·6	0·8
(c)	„ „ „ ..	5·0	4·8	3·6	5·0	4·4	3·8	..	..	..	5·0	2·2	1·0
4 (a)	„ „ „ ..	5·0	0·8	0·2	5·0	0·6	0·2	5·0	0·6	0·2	5·0	0·8	0·4
(b)	„ „ „ ..	5·0	3·6	2·8	5·0	3·0	2·1	5·0	4·0	3·6	5·0	2·2	0·8
(c)	„ „ „ ..	5·0	4·4	4·2	5·0	3·8	3·4	..	..	..	5·0	2·6	1·2

TABLE II.

The degree of protection exerted by tissue extracts against the catalytic oxidation of vitamin C by Cu¨.

Guinea-pig number.	Degree of protection.			
	Liver.	Kidney.	Adrenals.	Brain.
A. Normal guinea-pigs.				
1	50	40	75	8·3
2	55	28	33	8·3
3	60	58	67	6·3
4	38	31	79	16·6
B. Scorbutic guinea-pigs.				
1	70	30	30	15·0
2	83	42	58	0·0
3	75	46	80	8·7
4	55	40	67	8·7

The results presented in Tables I and II show that all the tissues—liver, kidney, adrenals, and brain—possess the protective action against the catalytic oxidation of vitamin C by C¨. The degree of protection exerted by brain is significantly lower than that of other tissues. Further, the results show that there are no significant differences between the protective action of tissue extracts of scorbutic and normal guinea-pigs.

B. The nature of the protective substances.

1. *The effect of dialysis of liver extract on the protective action against the catalytic oxidation of vitamin C by Cu¨.*—The aqueous extract was prepared as described previously from the liver of a guinea-pig fed on the scurvy-producing diet supplemented with 5 mg. of vitamin C per day.

Fifty ml. of the extract were dialysed in collodion bags for three days, with the addition of toluene. After dialysis, it was centrifuged. The clear extract was made up to 100 ml. and designated Ext. A. The precipitate obtained after centrifuging the dialysed extract was dispersed in 100 ml. water and this extract was designated as Ext. B.

Another 50-ml. sample of the liver extract was diluted to 100 ml. (Ext. C) and used without dialysis.

The protective action of the three liver extracts was determined, and the results are presented in Graph 1.

GRAPH 1.
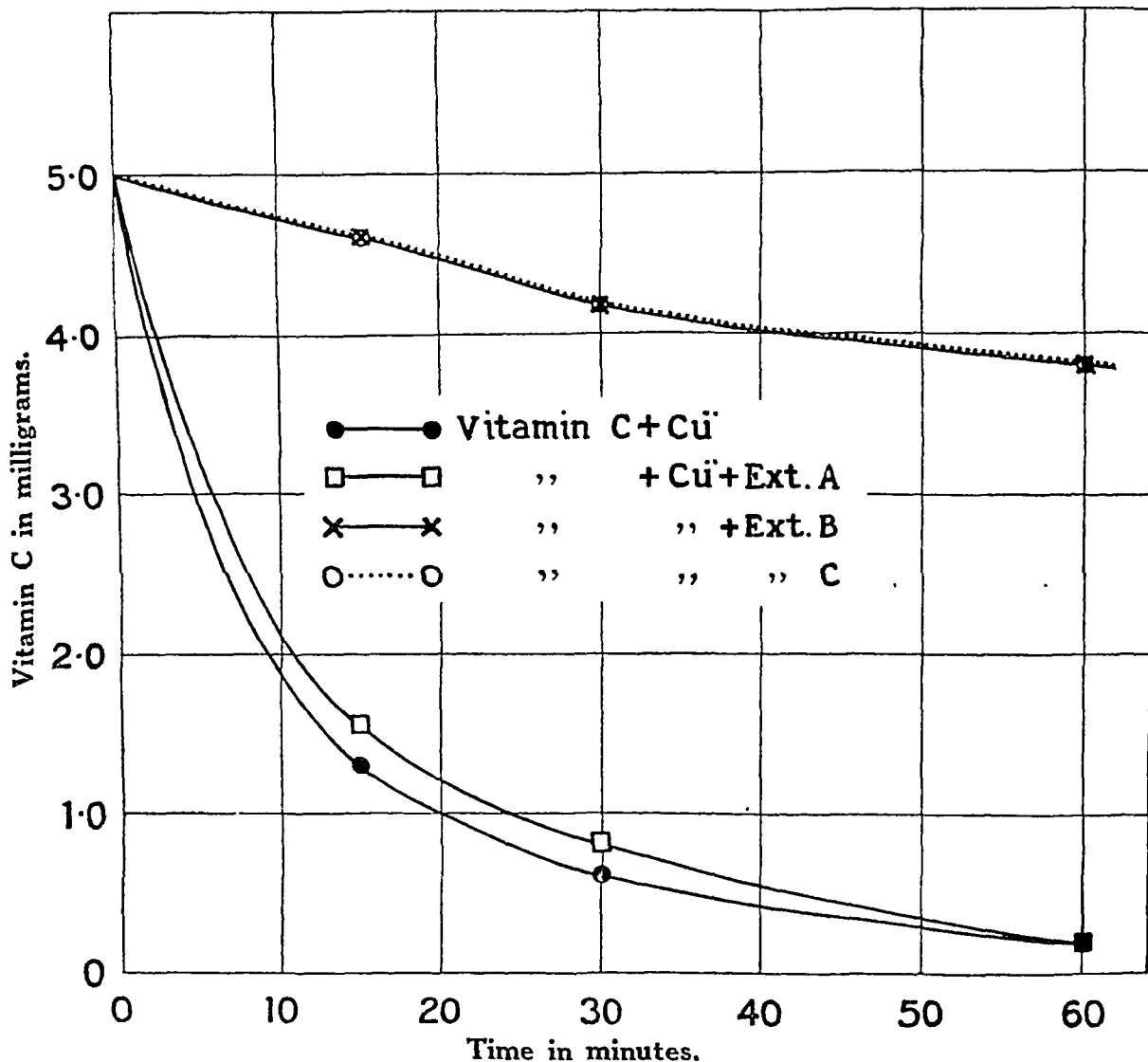
Effect of dialysis on the protective action of liver extract against the catalytic oxidation of vitamin C by Cu``.

Ext. A = (4 ml.) dialysed and centrifuged.
" B = " aqueous suspension of the precipitate after dialysis.
" C = " undialysed.

It may be seen from the figure that dialysed liver extract (Ext. A) after the suspended precipitate is removed by centrifugation, is devoid of protective

properties, while the suspended precipitate (Ext. B) obtained after centrifuging the dialysed extract retains in full the protective action of the original extract (Ext. C). It is clear, therefore, that the protective action of liver extracts is mainly confined to the undialysable and colloidal constituents of the liver.

GRAPH 2.
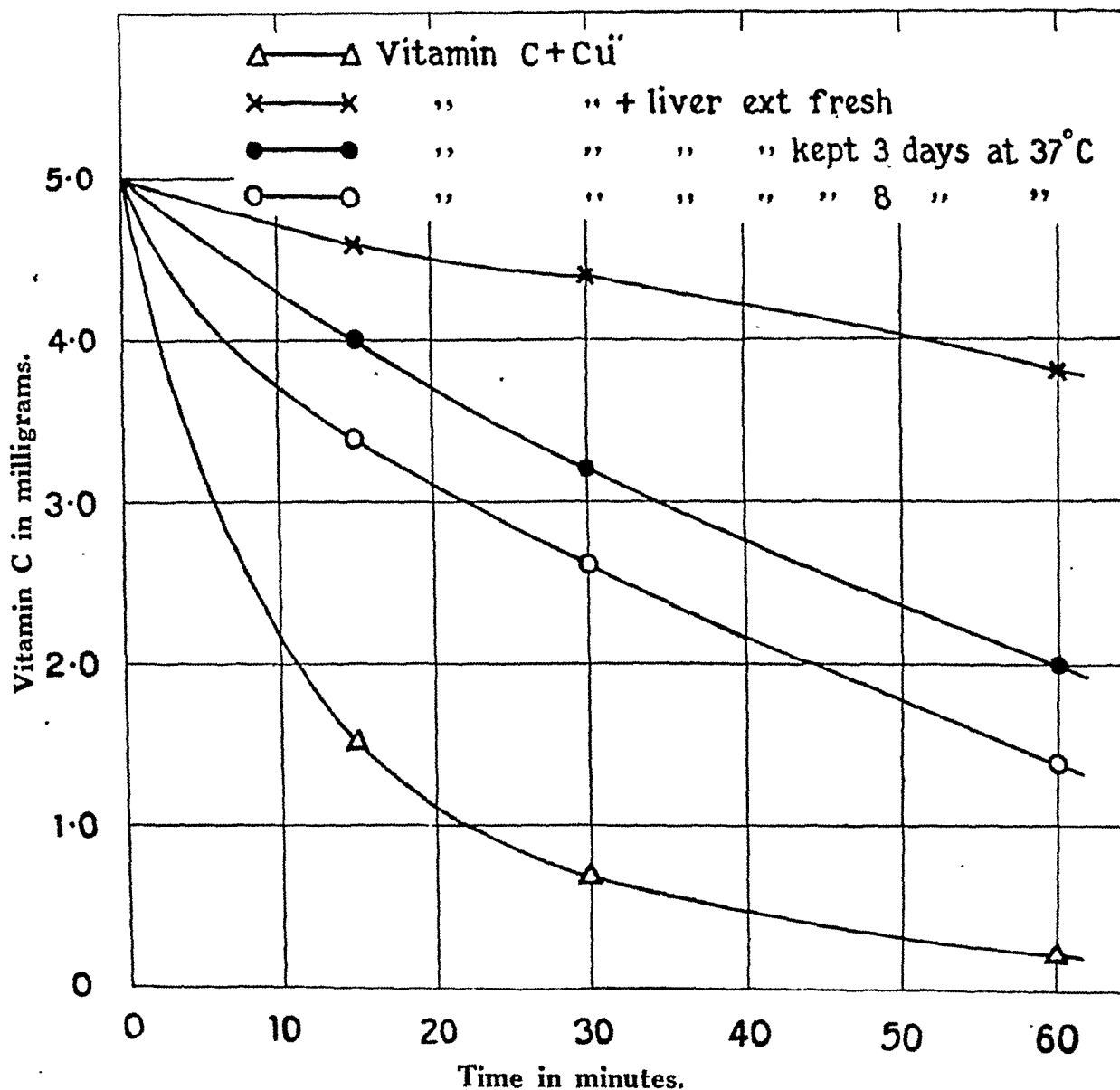
Variation in the protective action of liver extract on keeping at 37°C.

2. *Variation in the protective action of liver extract on keeping.*—The protective action of the extract was determined at stated intervals of time. The extract was

kept in an incubator at 37°C., with the addition of toluene. The results are presented in Graph 2.

The curves in Graph 2 indicate that the protective action of the extract decreases gradually on keeping.

Similar results were obtained when the aqueous extract of the precipitate (Ext. B) obtained after centrifuging the dialysed liver extract was kept at 37°C. After 4 days there was a considerable decrease in the protective action.

3. *The protective action of acetone extracts of liver.*—Sixteen grammes of the liver of a guinea-pig fed on a vitamin-C deficient diet plus a daily supplement of 5 mg. vitamin C were extracted with 80 ml. of acetone and kept over-night in an ice-chest. After filtration the acetone was removed by evaporation on a water-bath. The residue was taken up in 50 ml. of water, and the protective action of the aqueous extract determined. The results presented in Table III show that the residual material from the acetone extract possesses very feeble protective action, compared with that of the aqueous extracts of liver. Thus, the protective substances are extracted by acetone in negligible amounts.

TABLE III.

Protective action of the acetone extract of liver.

	Mg. vitamin C in the total volume of the reaction mixture. Time in minutes.		
	0	30	60
1. Vitamin C + Cu˙˙	5·0	0·8	0·4
2. Vitamin C + Cu˙˙ + 1 ml. of extract	5·0	1·0	0·4
3. Vitamin C + Cu˙˙ + 2 ml. of extract	5·0	1·6	0·6

DISCUSSION.

The experiments described show that all the tissues examined possess the protective mechanism against the catalytic oxidation of vitamin C by Cu˙˙. The degree of protection exerted by the brain extract was found to be much less than that of the adrenals, liver, and kidney. No significant differences were noticed

between the protective action of the phosphate-buffer extracts of the tissues of normal and scorbutic guinea-pigs. Thus, the protective mechanism of the tissues is not altered by the onset of scurvy. This finding is of physiological interest, as the existence of such a protective mechanism in the tissues of the scorbutic animals is essential for effecting the cure of scurvy by vitamin C.

Further analysis of the tissues of guinea-pigs has shown that the protective mechanism is mainly confined to the colloidal and undialysable constituents of the tissue extracts. On dialysing and centrifuging the phosphate-buffer extracts of the liver, the protective substances can be removed completely, leaving behind a clear aqueous solution, which retains the soluble enzymes. This method of separation may be useful for obtaining an enzyme extract free from protective substances for use in investigations on the influence of vitamin C on enzyme systems in the absence of such protective mechanism. The precipitate obtained on centrifuging the dialysed extract of liver retains all the protective substances originally present in the extract. Thus, it is clear that the protective mechanism is mostly confined to the colloidal aggregates of the extract. In a previous communication (Giri, 1939a) it was found that the aqueous extracts of tissues obtained after dialysing and centrifuging to remove the suspended impurities did not exert protective action. The suspended impurities which were removed after dialysis retain all the protective mechanism and their removal leaves a clear aqueous extract free from the protective substances. The present findings are therefore in conformity with the observations made in the previous investigation (Giri, 1939a, b) on the interaction of vitamin C and tissue phosphatases.

Little is known regarding the chemical nature of the protective mechanism. On keeping the aqueous extracts of liver at 37°C. the protective action gradually diminishes. It is possible that the protective substances are destroyed either by enzymic action or by some other chemical change taking place on autolysis. Very little of the protective substances could be extracted with acetone. Further work on the chemical nature of the protective substances is in progress.

SUMMARY.

1. The protective action of tissues of scorbutic and normal guinea-pigs against the oxidation of vitamin C has been investigated.

2. Liver, kidney, and adrenals, possess the protective mechanism to approximately the same extent, brain extracts being less effective. No significant differences were noticed between the protective action of the tissues of scorbutic and normal guinea-pigs.

3. The protective mechanism is mainly confined to the undialysable and colloidal constituents of tissue extracts. A method for the separation of the protective substances from the soluble enzymes has been evolved, which may be of use in investigating the influence of vitamin C on enzymes in the absence of the protective mechanism. The protective action of the extracts gradually diminished on keeping.

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THE EVALUATION OF THE INTRADERMAL DYE TEST FOR VITAMIN C IN HEALTH AND DISEASE.

BY

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IN 1937, Rotter showed that intracutaneous injections of 2 : 6-dichlor-phenol-indophenol into soles of guinea-pigs were decolorized by the tissues at a rate depending upon their vitamin-C contents. He obtained similar results in human subjects on vitamin-C rich and vitamin-C free diets and established that in 'saturated' cases the dye disappeared in less than 5 minutes, in normal cases in 5 to 10 minutes and in deficient cases in more than 10 minutes. Later, Portnoy and Wilkinson (1938) performed this test in 103 patients estimating at the same time the vitamin-C content of their blood and confirmed Rotter's findings that a prolonged decolorization time apparently parallels a deficiency of vitamin C. They concluded that although the intradermal test is not a direct measure of the vitamin-C content of the tissues since other reducing substances present in them, such as glutathione, will also cause a reduction of the dye, it is a useful and rapid clinical test for vitamin-C 'subnutrition' and worthy of further study. When Portnoy and Wilkinson's work was published we were already working on the vitamin-C content of blood in health and disease. The simplicity of the intradermal test attracted us and we decided to introduce this test along with the blood estimation. A few months

later Poncher and Stubenrauch (1938) in America published the result of their experience with the test in 41 cases and came to the conclusion that the intradermal test could not be relied on to give satisfactory clinical information about vitamin-C deficiency. The contradictory report of the American workers made us examine a much larger number of cases in order to ascertain its value. So far we have examined 140 students of the K. E. Medical College (ages between 19 and 21 years) belonging generally to the middle and upper middle classes and 200 in-patients suffering from various diseases in the Mayo Hospital, Lahore.

EXPERIMENTAL.

A 40-mg. per cent solution of 2 : 6-dichlor-phenol-indophenol used for intradermal dye test was prepared as follows : Hundred mg. of Merck's dye were dissolved in 100 mil. of water, filtered, and autoclaved. This was standardized by removing an aliquot portion of it with a sterilized pipette against a standard solution of vitamin C which in turn was freshly standardized against standard iodine solution. The standardized dye was diluted with sterilized water to make up 40 mg. per 100 mil. of the solution. The dye solution was freshly prepared every day from the stock solution (0·1 per cent) which in turn was titrated against freshly-prepared standardized vitamin C on every fourth day. Fresh stock dye solution was prepared every week.

A micro-syringe with which we could measure exactly 0·01 mil. of the dye with the help of a micrometer attachment was constructed by us but it was not only inconvenient to use in routine work but the resistance offered by the cutaneous tissue during the injection interfered greatly with the accuracy of this method. We preferred to produce instead a uniform epithelial wheal of the dye measuring 2 mm. as described by Portnoy and Wilkinson (*loc. cit.*). The site chosen was the front of the forearm which is devoid of hair and superficial veins. Four wheals were produced and the average time of their complete disappearance was determined. In a few of the cases the intradermal test was repeated on the same individual on three or four consecutive days and was found not to vary significantly. It is apparent, therefore, that there was no appreciate technical error in the performance of the test.

The vitamin-C content of the blood plasma was determined by titrating the protein-free plasma as follows : Two mil. of oxalated plasma taken in a centrifuge tube to which 4 mil. of 5 per cent freshly-prepared meta-phosphoric acid was added and centrifuged or filtered. Three mil. aliquot (1 mil. of plasma) of the filtrate was titrated immediately against freshly-standardized 0·002 per cent dye to a standard colour produced by the addition of 1 mil. of 6·5 per cent cobaltous chloride ($COCl_2,6H_2O$) and 0·15 mil. of 6·5 per cent copper sulphate ($CUSO_45H_2O$) to 15 mil. of distilled water. A blank titration with 2 mil. of 5 per cent meta-phosphoric acid and 1 mil. of water performed and brought to a pink coloration as described above. The titration value of the blank was deducted from the titration value of the unknown. All precautions were taken not to expose the plasma or the filtrate much to the air and the whole procedure was finished within one to one and a half hours. All glass distilled water was used throughout in order to avoid traces of copper which may act as catalyst.

In some of the samples of blood potassium cyanide was added before separating the plasma to inhibit the slow inactivation of vitamin C as suggested by Pijoan *et al.* (1937) but this procedure was abandoned as no difference was noted by its use.

RESULTS AND DISCUSSION.

Students.—The average vitamin-C content of the blood plasma and the decolorization time with mean standard deviation in 140 determinations on students were $1 \cdot 19 \pm 0 \cdot 3$ mg. per 100 mil. of blood plasma and $8 \cdot 2 \pm 4 \cdot 2$ minutes respectively. A statistical summary is given in Table I :—

TABLE I.

Vitamin-C level in blood plasma, mg. per cent.	Decolorization time in minutes.			More than 15.	Total number of cases.	Percentage.
	Less than 5.	5 to less than 10.	10 to less than 15.			
Less than 0·8 ..	..	6	7	7	20	14·3
0·8 to less than 1·5 ..	18	27	29	7	81	57·8
1·5 and more ..	28	6	5	..	39	27·9
Total number of cases ..	46	39	41	14	140	..
PERCENTAGE ..	32·8	27·9	29·3	10·0	..	..

Table I suggests that with low vitamin-C content the time taken for decolorization is large and vice versa. In order to test the significance of this fact statistically the expected frequencies for all the cells in the table were calculated and none was found to be very low. The value X^2 for this table was found to be 59·5. This value when referred to Fisher's tables for 6 degrees of freedom establishes a significant degree of association between the two variables. It is, therefore, statistically established that the decolorization time is not independent of the vitamin-C level and that the two are inversely related. A coefficient of correlation has been calculated

for all the 140 healthy students treated as one sample and the value obtained is —0·6522, which is about 7·8 times of its standard error of $1/\sqrt{140-1} = 0.084$. It

GRAPH 1.

Students.

Vitamin C, mg. per cent.
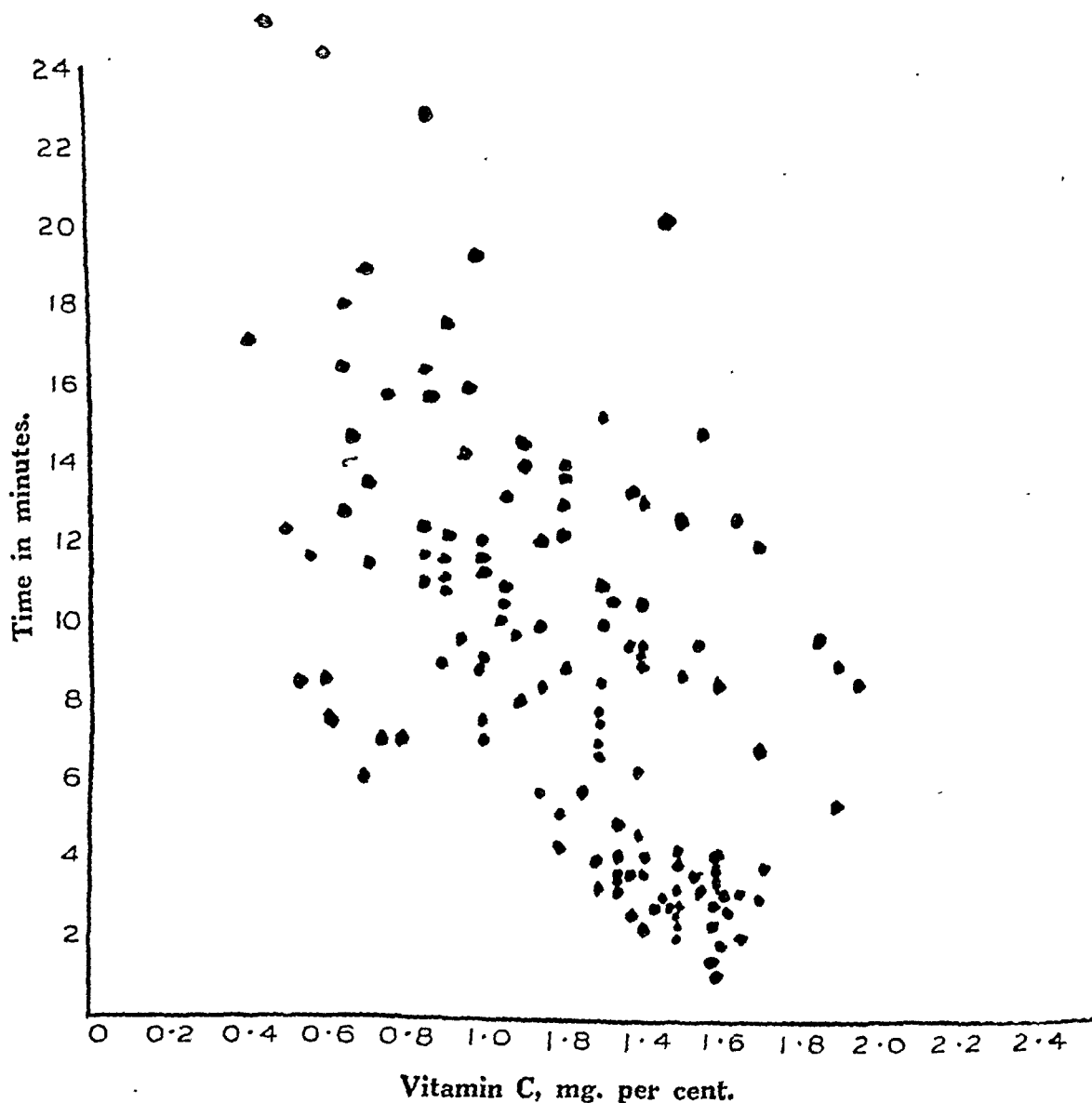
is concluded, therefore, that there is some degree of correlation between the two characters. But it is quite clear from the scatter diagram in Graph 1 that the

degree of correlation is not sufficiently high to make the test of any use in clinical practice. For instance a blood level of 1·30 mg. to 1·35 mg. per cent has a decolorization time of 3·4, 3·5, 3·6, 3·7, 4·1, 4·2, 5·0, 6·6, 7·0, 7·1, 8·6, 10·7, 11·0, and 15·3 minutes.

Table I shows that out of 140 apparently healthy students only 20, i.e., 13·6 per cent, gave figures for vitamin-C content of the blood below 0·8 mg. 100 mil. and are classed as sub-optimum (King, 1938).

In-patients.—The average vitamin-C content of the blood plasma in 200 determinations on in-patients was 0·88 ± 0·20 mg. per cent with 9·1 ± 3·6 minutes as average decolorization time. The degree of correlation between the decolorization time and the vitamin-C level in the blood plasma is represented in Table II :—

TABLE II.

Vitamin-C level in blood plasma, mg. per cent.	DECOLORIZATION TIME IN MINUTES.				Total number of cases.	Percentage.
	Less than 5.	5 to less than 10.	10 to less than 15.	More than 15.		
Less than 0·8 ..	2	32	19	7	60	30
0·8 to less than 1·5 ..	12	91	28	7	138	69
1·5 and more ..	..	1	1	..	2	1
Total number of cases ..	14	124	48	14	200	..
PERCENTAGE ..	7	62	24	7	..	..

Table II suggests that the vitamin-C content is not so much inversely related to the decolorization time as was in the case of students. The value X^2 for this table was 8·34. This value when referred to Fisher's table for 6 degrees of freedom gives a probability figure of 0·19 showing that the difference between the proportion of cases in each cell of this table might have arisen by chance and we can draw

from these no tentative conclusions. The value of coefficient of correlation for these 200 cases is —0·6111 which establishes a significant degree of correlation but

GRAPH 2,

In-patients.

Vitamin C, mg. per cent.
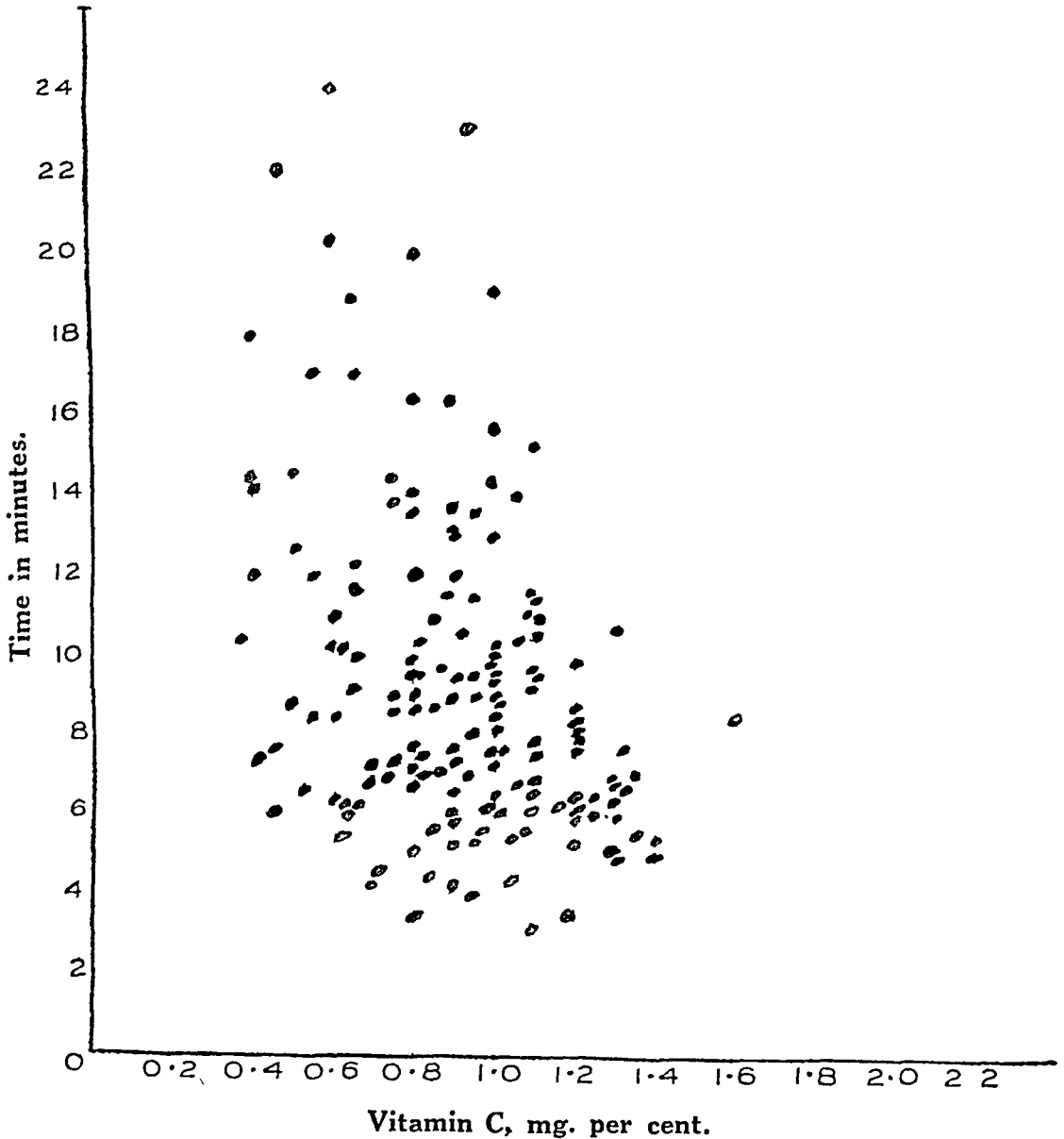
not so much as to establish the intradermal test as a measure of vitamin-C level in blood. Graph 2 proves this statement.

Table III gives a summary of results of vitamin-C level in blood plasma of 200 in-patients suffering from diseases mentioned therein :—

TABLE III.

Disease.	Number of cases.	Average vitamin C in blood, mg. per cent.	Average decolorization time in minutes.
1. Pneumonia	14	0·76 ± 0·17	7·9 ± 1·9
2. Muscular dystrophy	5	0·97 ± 0·13	13·4 ± 4·7
3. Diarrhœa and dysentery	6	0·76 ± 0·24	9·6 ± 4·0
4. Gastric and intestinal diseases	9	1·03 ± 0·19	9·0 ± 3·9
5. Heart failure	9	0·93 ± 0·22	8·3 ± 2·7
6. Bronchitis	11	0·86 ± 0·19	7·8 ± 4·1
7. Nervous diseases	8	0·87 ± 0·19	8·5 ± 2·4
8. Tuberculosis	60	1·05 ± 0·25	9·85 ± 4·3
9. Miscellaneous	79	0·72 ± 0·23	8·2 ± 2·8
TOTALS	200	0·88 ± 0·20	9·1 ± 3·6

A study of Table III confirms the findings of previous workers that vitamin-C content of the blood plasma is deficient in various infections, the only contradictory figures are those of the tuberculous patients but this may be due to the better appreciation of the value of vitamin C in tuberculosis and consequently an increased intake of this vitamin.

A comparison of Tables I and II shows that in the case of in-patients the decolorization time does not bear the same relation to vitamin-C content of the blood plasma as in the case of students. In the former case the vitamin-C content was mostly either on the lower limit of normal or in the sub-optimum level and the decolorization time was mostly between 5 and 10 minutes (62 per cent of the total cases), whereas in the case of students with the same level of vitamin C the decolorization time was equally distributed, i.e., 32·8 per cent in less than 5 minutes, 27·8 per cent in 5 to 10 minutes, and 29·3 per cent in 10 to 15 minutes. This wide variation in the decolorization time of healthy and diseased subjects when the vitamin-C content of the blood is equal is difficult to explain.

In the case of students suffering from pyorrhœa, chronic tonsillitis, and defective vision, blood vitamin-C values did not differ significantly from those of

the healthy students (Table IV). A number of cases of pyorrhœa in the hospital were also tested for vitamin-C subnutrition but the low figures in the hospital cases (Table V) might be due to the other co-existing diseases such as pneumonia, bronchitis, diarrhœa, etc. Further work on the relation of pyorrhœa to vitamin-C subnutrition is in progress.

TABLE IV.

Showing vitamin-C content of blood with decolorization in students with pyorrhœa, tonsillitis, and defective vision.

	Number of students.	Average vitamin C in blood, mg. per cent.	Significance, difference.	Average decolorization time in minutes.	Significance, difference.
TOTALS :—	140	1·19	0·30	8·2	4·2
Pyorrhœa	19	1·14	0·27	9·8	3·4
Chronic tonsillitis ..	11	1·30	0·25	10·2	2·7
Defective vision ..	18	1·14	0·42	11·3	3·6

This table shows no significant difference. Therefore no correlation was found in medical students between the vitamin-C content of the blood in normal subjects and in those with pyorrhœa, chronic tonsillitis, and defective vision.

TABLE V.

Showing comparative vitamin-C content of blood with decolorization time in pyorrhœa cases.

	Number of cases.	Average vitamin C in blood, mg. per cent.	Average decolorization time in minutes.
Pyorrhœa in student ..	19	1·14 ± 0·27	9·8 ± 3·4
Pyorrhœa in healthy but poorly-paid people.	15	0·95 ± 0·25	9·8 ± 2·6
Pyorrhœa in patients ..	27	0·69 ± 0·23	7·4 ± 2·5

Summary.

1. The intradermal vitamin-C test was performed in 140 apparently healthy students and 200 patients.

2. Rotter's finding that the vitamin-C content of the tissues is comparable to the decolorization time in guinea-pigs is in our experience not true for human beings in health and disease.

3. In our present state of knowledge we possess no reliable test for estimating vitamin-C level in human subjects except the determination of the vitamin-C content of the plasma.

4. Sub-optimum values of vitamin-C content of the plasma is not common among otherwise healthy college students.

5. Our findings agree with other workers that various infections cause some deficiency of vitamin C in the body, thus necessitating increased intake of this vitamin.

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THE TREATMENT OF EYE, SKIN, AND MOUTH LESIONS DUE TO VITAMIN DEFICIENCIES.

BY

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INTRODUCTION.

LESIONS of the eyes, skin, and mouth have been widely used in recent years in India, Ceylon, and other tropical countries, for the assessment of malnutrition.

Bitot (1863) observed white spots in the conjunctiva of a number of orphans admitted for night-blindness to a Foundling Hospital in Bordeaux, these have since been called Bitot's spots. He stated that the spots were invariably associated with night-blindness; but this association has not occurred in the majority of our cases.

Both these eye changes are now known to be due to a deficiency of vitamin A.

Aykroyd (1930) studied seasonal outbreaks of night-blindness among fishermen in Newfoundland and Labrador.

Xerophthalmia and keratomalacia had frequently been recorded among malnourished children long before animal experiments showed that they were due to vitamin-A deficiency. In recent years these lesions of the eye have been shown to be common among the poorer classes in China (Pillat, 1929), in India (Wright, 1931), and in Ceylon (Nicholls, 1933).

Pillat (*loc. cit.*) was the first to describe skin changes in adult Chinese suffering from xerophthalmia. Lowenthal (1933*a, b*) and Nicholls (1933) described similar skin lesions among prisoners in Uganda and Ceylon. The latter also observed

(705)

that keratomalacia, neuritis, and persistent diarrhœa, often accompanied them and accepted vitamin-A deficiency as the cause of the papular eruption which he named 'phrynoderma'.

Nicholls (1934) showed the high incidence of sore-mouth among malnourished children and pointed out the inspectional value of sore-mouth and phrynoderma in assessing the state of nutrition of children (Nicholls, 1934, 1935).

Aykroyd and Krishnan (1936) described these conditions among South Indian school children who were receiving milled parboiled rice, a little dhal or gram, few vegetables with little or no meat or milk. They showed that cod-liver oil had no effect on sore-mouth but that 1 oz. dried brewer's yeast given daily cured it in less than 4 weeks, while 1½ oz. dried skim milk was equally effective. In a further paper they suggested that the anti-stomatitis vitamin was the same as the pellagra-preventive factor of vitamin B_2 (Aykroyd and Krishnan, 1937).

Landor (1939) has shown that, while dried autoclaved yeast is effective, nicotinic acid is not, in the treatment of sore-mouth.

THE SURVEY OF A HOSTEL FOR INDIGENT CHILDREN.

There were 104 girls in the hostel in question, and the incidence of signs of dietary deficiencies was very high among them. Their daily diet consisted of polished parboiled rice, a little meat or fish, a vegetable, bread, and tea with a little sweetened condensed milk. Dhal or gram was given two or three times a week. Table I gives the incidence of the various signs :—

TABLE I.

Clinical sign.	Number.	Percentage.
Dry skin	68	65·4
Phrynoderma	33	31·7
Angular stomatitis	39	37·5
Erosin of tongue	15	14·4
Xerophthalmia	20	19·2
Bitot's spots	6	5·8
Marked anæmia	15	14·4

Of the 104 children examined 25 showed one clinical sign, 22 showed two, 21 showed three, while 15 showed four or more than four clinical signs. There were no signs in 21 children.

EXPERIMENTAL TREATMENT.

Twenty-five children showing, among other signs, phrynoderma and sore-mouth, were selected and divided into five groups. One group was kept as a control, and others were treated with Haliverol, gingelly seeds, and yeast extract, or combinations of these. The children were examined weekly.

Group I.—These children were given capsules of Haliverol one daily, and from the 24th day of the experiment they were also given a drachm of a liquid extract of yeast twice a day. Each capsule of halibut-liver oil contained 9,400 I.U. vitamin A and 1,700 I.U. vitamin D.

Group II.—These children were given slabs of a preparation of gingelly seeds and jaggery. This was tried because certain unsaturated fatty acids are known to be essential for healthy skins in rats. The fats of the ordinary diets of these children were supplied mainly by coco-nut oil which is very deficient in unsaturated fatty acids. Gingelly seeds are rich in linoleic acid (Hoover, 1939) and were given to test the effect of this 'essential' fatty acid on the skin lesions. The two slabs which were given daily contained 1 g. linoleic acid and 0·4 g. calcium (gingelly seeds contain some carotene). These children were also given extract of yeast from the 24th day.

Group III.—Children of this group were given both Haliverol and the gingelly preparation.

Group IV.—These children were given an extract of yeast, a teaspoonful twice a day. It was prepared in the manner given below.

Group V.—These children were kept as a control group.

The diet of the whole institution was unaltered during the period of the experiment.

PREPARATION OF YEAST EXTRACT.

Half a litre of rectified spirit was poured on 2 lb. of broken up yeast cake, and this was thoroughly stirred and allowed to stand for 24 hours and the alcohol removed by centrifuging. Three hundred c.c. of alcohol were stirred into the deposit and centrifuged off after six hours.

The yeast was similarly treated with 500 c.c. and 300 c.c. of water. The combined alcohol and water extract was evaporated down to 800 c.c.

Glycerine, benzoic acid, and oleum anisi, were added to this concentrated extract in accordance with the following formula :—

Concentrated yeast extract	..	..	800 c.c.
Glycerine	..	..	40 c.c.
Benzoic acid	..	..	2 g.
Oleum anisi	..	..	7 c.c.

The dose was one teaspoonful twice a day. This preparation proved to be a convenient form for giving yeast, the added ingredients preserved it from decomposition and the children found it palatable.

RESULTS.

Group I.—The skin and eye lesions of all five children of this group responded quickly to the Haliverol treatment. The phrynoderma was markedly less by the 24th day. There was no effect on the signs of sore-mouth by the 24th day; they were then given the preparation of yeast, and the sore-mouths quickly responded and had completely cleared by the 42nd day. Table II gives the results:—

TABLE II.

Clinical sign.	No. 1 7 YEARS. Before experiment.	24th day of experiment.	42nd day of experiment.	No. 2 9 YEARS. Before experiment.	24th day of experiment.	42nd day of experiment.	No. 3 11 YEARS. Before experiment.	24th day of experiment.	42nd day of experiment.	No. 4 14 YEARS. Before experiment.	24th day of experiment.	42nd day of experiment.	No. 5 15 YEARS. Before experiment.	24th day of experiment.	42nd day of experiment.
Dry skin	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−
Phrynoderma	+	im	−	+	im	−	+	im	−	+	vl	−	+	im	−
Angular stomatitis.	+	+	−	+	+	−	+	+	−	+	+	−	+	+	−
Eroded tongue	+	+	−	−	+	−	−	+	−	+	+	−	+	+	−
Xerophthalmia	+	+	−	+	−	−	−	−		−	−		−	−	−
Bitot's spots	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−

im = some improvement.
vl = very little left.

Group II.—The dry skins of these children taking gingelly seeds improved in four cases out of five; but the phrynoderma did not improve; and although there was some improvement in one case out of three in xerophthalmia, it must be

concluded that neither xerophthalmia nor phrynoderma are affected by linoleic acid. The mouth signs improved as soon as the extract of yeast was given. Table III gives the results :—

TABLE III.

Clinical sign.	No. 6 7 YEARS.			No. 7 9 YEARS.			No. 8 11 YEARS.			No. 9 8 YEARS.			No. 10 15 YEARS.		
	Before experiment.	24th day of experiment.	42nd day of experiment.	Before experiment.	24th day of experiment.	42nd day of experiment.	Before experiment.	24th day of experiment.	42nd day of experiment.	Before experiment.	24th day of experiment.	42nd day of experiment.	Before experiment.	24th day of experiment.	42nd day of experiment.
Dry skin ..	+	—	—	+	—	—	+	—	—	+	—	—	+	—	+
Phrynoderma ..	+	+	+	+	+	—	+	+	+	+	+	+	+	+	+
Angular stomatitis.	+	+	—	+	+	vl	+	+	vl	—	+	—	+	+	—
Eroded tongue	+	+	vl	—	+	—	—	+	—	+	+	—	—	+	—
Xerophthalmia	+	+	+	+	+	+	—	—	—	—	—	—	+	—	—
Bitot's spots ..	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—

vl = very little left.

Group III.—The skin and eye lesions of these children on Haliverol and gingelly seeds healed rapidly. The sore-mouth was uninfluenced by the treatment and, after 42 days, four out of the five were still suffering from it. These children were not given yeast extract.

Group IV.—The dry skin and phrynoderma of all five children of this group remained unchanged at the end of 42 days' treatment with yeast extract. The sore-mouths of all five healed within three weeks. Three children of this group were affected with xerophthalmia, and at the end of the experiment there was improvement in one case.

Group V.—These children were kept as a control. Their condition remained more or less the same at the end of the experiment.

Nicotinic acid.

Swaminathan (1938) estimated the amount of nicotinic acid in foodstuffs. His estimates were approximately 60 mg. per cent for dried brewer's yeast and 10·9 mg. per cent for skimmed milk powder. From these determinations the amounts of nicotinic acid in the 1 oz. of yeast and the $1\frac{1}{2}$ oz. of skimmed milk powder which Aykroyd and Krishnan found effective in curing 'sore-mouth' in children were roughly 16 mg. and 5 mg. daily, respectively. The amount of nicotinic acid in the yeast extracts we gave to the school children was less than 6 mg. daily.

We tested the effect of nicotinic acid on prisoners with sore-mouth. Twenty prisoners were selected; ten were given 90 mg. nicotinic acid daily and ten were used as controls. At the end of 15 days no improvement had taken place in the treated prisoners. In this period of time the sore-mouths of all the children who were given yeast extract had either cleared up or markedly improved. This lends some support to the conclusions of Landor.

Katzenellenbogen (1939) found nicotinic acid in daily doses of 200 mg. effective in the treatment of glossitis in Palestine. These were probably early cases of pellagra but there is no evidence that the commonest type of 'sore-mouth' of India, Malaya, and Ceylon, has the same aetiology.

CONCLUSIONS AND SUMMARY.

Dry skin and phrynoderma responded to treatment with vitamin A. Linoleic acid had no curative effective on phrynoderma. Sore-mouth (angular stomatitis and erosion of tongue) responded to treatment with extract of yeast, but nicotinic acid had no effect.

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VITAMIN-A CONTENT OF LIVER AND DEPOSIT FATS
OF SOME INDIAN FISH.

BY

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INTRODUCTION.

THE preparation of fish-liver oils for medicinal purposes from Indian fish has not received sufficient attention. With an extensive sea surrounding the three sides of the vast peninsula, India has a large and inexhaustible source of marine fish. In addition, such large rivers as the Ganges, the Brahmaputra, the Indus. the Godaveri, the Kistna, and the Cauvery, contain a large amount of fresh-water fish. Though some of these fish are commonly consumed, there are several varieties which are not popular for edible purposes. At present fish oils are prepared in India mainly for use as fat and lubricants. The nutritional values of the Indian fish oils, particularly as sources of vitamins A and D, have not been adequately studied. It is well known that the vitamin reserve of fish shows a remarkable species variation and considerable fluctuation during season and growth. Chakravorty *et al.* (1933), Ghosh *et al.* (1933), Datta and Banerjee (1934), Ghosh and Guha (1935), and Basu (1938) have examined the vitamin-A content of oils of some of the popular fish consumed in Bengal by tintometric and biological methods of assay. De *et al.* (1938) have determined the vitamin-A content of the oils of fish found in Madras by spectrophotometric methods. All the workers have observed that certain of the liver oils prepared were rich in vitamin A. The vitamin-A content of the liver and deposit fats of 16 different species of fish have been determined, some marine and some fresh-water, in two different seasons by both spectrophotometric and tintometric methods of assay.

MATERIALS STUDIED.

Only those species of big fish which yielded an appreciable quantity of oil were chosen. Since they were purchased from the local market a large number of species, particularly the marine ones, could not be obtained. Livers of shark,

saw-fish, and bat-ray were supplied through the Fisheries Expert to the Government of Bengal and Directors of the Indian Trawling Company, Limited. They were obtained from fish caught in a trawl net at a depth of 20 to 30 fathoms in the Bay of Bengal. The standard Norwegian cod-liver oil and the halibut-liver oil were kindly supplied by the Bengal Immunity and Parke, Davis & Co., respectively.

<p style="text-align:center">EXPERIMENTAL METHODS.</p>

The standard method recommended by the International Conference of Vitamin Standardization of the League of Nations' Health Organization (1934) for the assay of vitamin A, is the spectrophotometric estimation of the intensity of absorption band at 3280Å by a 1 per cent solution of the vitamin concentrate—non-saponifiable portion of the oil—in cyclohexane in a 1 cm. cell. This extinction coefficient (E value) multiplied by a conversion factor equal to 1600 gives the vitamin-A content of the oil per gramme. The accuracy of this conversion factor has again been confirmed recently (Hume, 1939) by the sub-committee of Lister Institute and Medical Research Council.

The apparatus that was used by us for the determination of the E value was the sector spectrophotometer supplied by Carl Zeiss. The spectrograph is a medium quartz instrument with a dispersion of 20Å per mm. at 3200Å. The photometric equipment consisted of a Hüfner prism to which can be attached two cells of any length from 1 mm. to 10 cm. The cells were made after the Scheibe pattern with detachable quartz windows. The rotating sector was graduated both in percentage ratio and density scales. The source of light was a high voltage condensed spark (12,000 volts, 10,000 cm. capacity) between electrodes of tungsten steel and brass, the spark-gap being 2 mm. to 3 mm. long. The high voltage was supplied by an oil-immersion transformer with an auto-transformer designed in these laboratories giving an output of 15,000 volts at 50 amps. The air-cooled leaky coil-type of transformer supplied by Carl Zeiss was found unsuitable for a moist climate prevailing in Calcutta for a major part of the year. Ilford Selochrome plates (H and D 1500) were used. The absorption spectra of the solutions of the oils and their concentrates in cyclohexane were photographed with suitable cells and concentrations (*vide* Morton, 1938; Twyman and Allsopp, 1934). The cyclohexane was freed from benzene by distillation after shaking with H_2SO_4. Twelve exposures of varying density from 0·3 to 1·4 or 50 to 4 per cent transmission were taken suitably increasing the time of exposure, in such a way that the time of exposure multiplied by the percentage of transmission was a constant, so that the comparison spectra were always photographed with equal intensity. The copper arc and the scale were photographed in each plate and the intense line of copper at 3274Å helped considerably in measuring the extinction co-efficient at 3280Å. The match-points were made visually and were found to be satisfactory. The extinction co-efficient was calculated for a 1 per cent solution and a 1 cm. cell. The collimation and arrangement of the apparatus enabled us to photograph the complete spectra in 2 minutes, the initial exposure being 2 seconds for 50 per cent transmission (0·3 density) and the longest 25 seconds for 4 per cent transmission (1·4 density). Since the photographs were taken in such a short interval the loss of vitamin A due to irradiation would be negligible.

The vitamin-A content of all the oils was also determined by the well-known Carr-Price method, measuring the blue colour produced by addition of $SbCl_3$ in chloroform using a Lovibond tintometer (Carr and Price, 1926 ; Coward *et al.*, 1931, 1932).

PREPARATION OF THE OILS.

The oils were prepared by the following methods and their vitamin-A content determined spectrophotometrically :—

(1) The livers were minced in a meat-mincer and mixed with dry sodium sulphate till they became a pasty mass. The whole mass was extracted with ether in a cool dim-lit laboratory in a closed bottle, vigorously shaking it for about half an hour. The extraction was repeated with fresh ether 3 or 4 times. The whole ether extract was collected and evaporated in a cool dark-room at room temperature, viz., 20°C. to 30°C. The oil was collected and stored in a coloured bottle in a frigidaire. Most of the ether from the ether extract from the above method was also reclaimed by distillation at 50°C. using a carbon-filament lamp. The temperature of the oil was never allowed to rise above 50°C.

(2) The livers were cooked in a large volume of water for $\frac{1}{2}$ to 1 hour and the oil that collected on the top was separated and dried over anhydrous sodium sulphate.

(3) The livers were tied in a cloth and placed in an atmosphere of steam at 100°C. for 15 minutes. The oil that separated out was collected and cooled to 0°C.

(4) The livers were placed in an air-oven at 70°C. to 90°C. for 15 to 30 minutes and the oil separated through a funnel.

(A) Rohit-liver oil.

		$E\frac{1\%}{1\,cm.}$ value.
(1) Cold ether extracted and evaporated at 20°C.	..	2·1
(2) Cold ether extracted and distilled at 50°C.	..	2·0
(3) Heated to 70°C. for $\frac{1}{2}$ hour and ether extracted	..	1·0
(4) Heated to 80°C. for $\frac{1}{2}$ hour in an air-oven	..	0·4

(B) Saw-fish-liver oil.

(1) Cold ether extracted and evaporated at 20°C.	..	5·2
(2) Cold ether extracted and distilled at 50°C.	..	5·0
(3) Heated in an air-oven for $\frac{1}{2}$ hour at 80°C.	..	3·0

(C) Shark-liver oil.

(1) Cold ether extracted and evaporated at 20°C.	..	0·75
(2) Heated in an air-oven for $\frac{1}{2}$ hour at 70°C.	..	0·48
(3) Cooked in water for $\frac{1}{2}$ hour at 100°C. ..	..	0·51
(4) Steam autoclaved for $\frac{1}{2}$ hour at 100°C. ..	..	0·20

In most of the cases the livers were not large and the oil could be collected only by ether extraction. From the above results, it can be found that the cold ether extracted oils show the highest E value, and that the heat treatment of the livers decreases the E value considerably. In order to retain all the vitamin-A reserve of the liver in the oil, the oils were all prepared by cold ether extraction. In some cases the ether was distilled at low temperature.

<div align="center">RESULTS.</div>

(a) *Vitamin-A content of the liver oils.*—Table I gives the vitamin-A content of liver oils of different species, some of them prepared in two different seasons. The International Units per gramme given in column 8 were obtained from the $E \frac{1\%}{1 \text{ cm.}}$ value using the conversion factor 1600 :—

<div align="center">TABLE I.</div>

<div align="center">*Vitamin-A content of liver oils.*</div>

Date of catch.	Local name of the fish.	Zoological name.	Occurrence of the fish.	Oil per liver in c.c.	Colour of the oil.	C.-P. blue units.	Int. units per gramme.	C.-P. value. / E value.
Norwegian	Cod	*Gadus callarius*	Marine	..	Light yellow	9	800	18
Parke Davis	Halibut	*Hippoglosus vulgarus*	,,	..	Yellow	1,000	48,000	33
24–2–39	Arh	*Mystus macronus*	Fresh-water	3	,,	800	44,800	29
24–2–39	Dhain	*Silonia siluroid*	,,	2·5	Light brown	700	38,400	29
13–1–39	Bôal	*Wallago attu*	,,	4	Brown	440	20,800	33
3–2–39	,,	,,	,,	4	,,	375	19,200	31
25–2–39	Shôle	*Ohiocephalus stratus*	,,	5	Dark brown	500	20,000	38
5–12–38	Shillang	*Silonia silompia*	,,	3	,,	350	22,400	25
10–1–39	,,	,,	,,	3	,,	200	12,800	25
29–3–39	Saw-fish	*Pristis species*	Marine	800	Yellow	160	9,900	26
5–10–38	Bhetki	*Lates calcifer*	Fresh-water	7	Brown	120	7,300	26
18–1–39	,,	,,	,,	8	,,	175	8,600	32
5–10–38	Mrigal	*Cirrhina mrigala*	,,	3	Yellow brown	100	5,100	30
27–1–39	,,	,,	,,	10	,,	120	8,800	22
13–1–39	Pangash	*Pangasius pangasius*	,,	4	Brown	130	7,200	30

TABLE I—*concld.*

Date of catch.	Local name of the fish.	Zoological name.	Occurrence of the fish.	Oil per liver in c.c.	Colour of the oil.	C.-P. blue units.	Int. units per gramme.	C.-P. value. / E value.
16–1–39	Kalbôs	*Labeo kalbasu*	Fresh-water	4	Brown	130	7,200	29
22–9–38	Shark	*Scolidon gangarius*	Marine	200	,,	90	4,600	31
22–11–38	,,	,,	,,	100	Red brown	80	4,300	30
24–11–38	,,	,,	,,	20	Light yellow	30	1,600	30
29–9–38	Rôhit	*Labeo rohita*	Fresh-water	10	Yellow	25	3,200	12
8–1–39	..	,,	,,	16	Brown yellow	25	3,500	12
5–10–38	Katlā	*Catla catla*	,,	3	Brown	15	2,200	10
5–2–39	..	,,	,,	2	Light brown	20	2,400	10
11–10–38	Ilish	*Hilsha ilisha*	Esturine	3	Yellow	4	300	20
12–1–39	,,	,,	,,	6	Brown	30	2,400	20
6–3–39	Chital	*Netopterus chitala*	Fresh-water	3·5	Yellow	14	1,900	12
4–11–38	Chingree	*Palimon carcinus*	Prawn	0·5	Red	10	1,400	10

(b) *Vitamin-A content of deposit fats.*—In most of the fishes purchased there was a large amount of peri-hepatic, peri-renal, and omental fat. This was also extracted by cold ether and yielded about 100 c.c. to 200 c.c. of oil per fish. In view of the large quantity of the oil available from this source, its vitamin-A content was determined. The oils from deposit fats were less viscous than the liver oils and were all coloured light yellow. Most of the oils gave no appreciable blue colour even in concentrated solutions on addition of the $SbCl_3$ reagent. Only a white or brownish precipitate was formed. Chital, Shôle, Bôal, and Rôhit, deposit fats showed faint blue colour (4 to 1 units) and the rest gave precipitates and various colours. Since the Carr-Price test could not be observed they were not examined spectrographically. It may be concluded that the vitamin-A content of these oils was quite negligible. Previous authors have observed that the body oils of the fish examined did not contain appreciable amount of vitamin A.

These observations are important in the preparation of the liver oils. The liver must be carefully separated from the adjoining body fats before extraction as the addition of these fats, though increasing the quantity of the oil, would decrease considerably the vitamin-A content.

(c) *Absorption curves of the liver oils.*—Recently considerable attention is given to the absorption-spectra curves of the fish-liver oils as these give

additional information regarding the chemical constituent of the oil. Edisbury *et al.* (1938), Grillam *et al.* (1938), and Lederer *et al.* (1938) have observed that in liver oils from fresh-water fish the peak of the absorption band is shifted to the longer wave-lengths compared to the marine ones. It has been suggested that this may be due to the presence of a higher homologue of vitamin A in the fresh-water fish-liver oils. The marine fish-liver oils show an absorption band with a maxima at 3280Å, while those of the fresh-water fish-liver oils are between 3300Å and 3500Å. In addition to this some often show an additional band at 2850Å. Though the origin of this band is not yet clearly known, opinion is gaining ground that this may be due to the presence of vitamin D (Lovern, Morton and Ireland, 1939). We have studied the absorption-spectra curves of the fish-liver oils prepared by us. Though all show very prominently an absorption band between 3800Å and 3000Å, some of them show an additional inflexion or at times a well-developed band between 3000Å and 2600Å. The correct position of the maxima and the E value at these wave-lengths are given in Table II :—

TABLE II.

Absorption bands of liver oils.

Fish.		Position of maxima, Å.	Conc., per cent.	Cell, cm.	Observed density.	$E \frac{1\%}{1\,cm.}$ value.
Cod	..	3,260	1·23	1·0	0·7	0·55
Halibut	..	3,280	0·089	0·5	1·3	29·1
Arh	..	3,370	0·042	0·5	0·6	28·5
,,	..	2,830	0·042	0·5	0·5	24·0
Dhain	..	3,400	0·045	1·0	1·1	24·4
,,	..	2,840	0·045	1·0	0·5	11·1
Bôal	..	3,350	0·088	1·0	1·1	12·9
,,	..	2,830	0·088	1·0	0·7	8·0
Shôle	..	3,300	0·087	1·0	1·1	12·6

TABLE II—*concld.*

Fish.	Position of maxima, Å.	Conc., per cent.	Cell, cm.	Observed density.	$E \frac{1\%}{1 \text{ cm.}}$ value.
Shôle	2,780	0·087	1·0	0·7	8·0
Shillang	3,240	0·055	1·0	0·8	14·5
Saw-fish	3,200	0·265	1·0	1·3	5·2
Bhetki	3,240	0·38	1·0	1·3	4·6
,,	2,730	0·38	1·0	0·8	2·7
Mrigal	3,240	0·38	1·0	1·2	3·2
,,	2,780	0·38	1·0	0·5	1·3
Pangash	3,280	0·279	0·6	0·8	4·5
Kalbôs	3,200	0·264	1·0	1·2	4·5
Shark	3,280	0·315	1·0	0·9	2·9
,,	2,920	0·315	1·0	0·9	2·9
Rôhit	3,280	0·396	1·0	0·9	2·3
Kätlä	3,240	0·37	1·0	0·5	1·4
,,	2,780	0·37	1·0	0·6	1·6
Ilish	3,300	0·58	1·0	0·9	1·5
,,	2,790	0·58	1·0	0·8	1·4
Chital	3,280	0·66	1·0	0·8	1·2
Chingree	3,280	0·604	1·0	0·5	0·9

From the above results we find that Arh, Dhain, Shôle, Bhetki, Mrigal, Kātlā, Ilish, and shark. show two absorption bands, the E value of the one at 2800Å always lesser than that of 3300Å band. Among these, shark alone is a marine fish and Ilish is a habitat of both fresh and marine waters. Whether the additional inflexion at 2800Å and the shift of the band to the longer wave-lengths are due to vitamin D or A₂ can be studied only by spectrophotometric studies on the blue colour produced by $SbCl_3$ reagent ; vitamin A_2 gives a band at 6900Å, while A_1 gives one at 6200Å. The relative intensity ratio of the bands at 6900Å and 6200Å gives the ratio of these components. Experiments on these lines are contemplated.

Some of the oils, particularly Dhain, Shôle, Shillang, and bat-ray, are very dark coloured and a solution of it intensely yellow. The absorption spectrum of this yellow solution resembles closely that of carotene (Morton, 1935). These pigments are completely soluble in petroleum ether and not in water. They are, probably, carotenoid pigments and are present in these oils in appreciable amounts in addition to the vitamin A.

In Plates XXXIII and XXXIV are given the contact prints of the absorption-spectra photographs of some of the liver oils taken with suitable concentrations and cells as given in Table II, to show the different nature of the curves, with particular reference to the inflection and band at 2850Å. The last picture in Plate XXXIV reproduces the absorption spectrum of bat-ray-liver oil in the visible and near ultra-violet taken with a Zeiss tungsten-filament lamp with Quartz window and a Baly's absorption tube.

DISCUSSION.

From the results and the observations of other workers it can be gathered that if fish-liver oils are prepared carefully avoiding all possible sources of loss, oils several times richer than cod and nearly comparable to halibut in their vitamin-A content can be prepared in India.

The oils obtained in Madras by the Fisheries Department were all prepared by cooking the livers in water in an open vessel. Our results and the experience of several others (Robinson, 1938) show that there is a considerable loss of the vitamin A when the oil is heated or cooked in contact with air. But some of the oils prepared by this method which causes more than 50 per cent loss were very rich in vitamin A. Of the 13 different oils examined 4 of them showed vitamin content, higher than cod. The vitamin-A content of some of the shark oils fluctuated from 8,000 to 24,000 International Units per gramme. The liver oils examined by tintometric and biological methods by previous workers were prepared by cold ether extraction. Since most of the liver oils gave a Carr-Price value higher than 100 it can be gathered that the vitamin-A content was more than 5 times that of cod.

Regarding the influence of season and growth of the fish, the observation on the vitamin-A content and the fat yield of 8 different species of fish included in Table I are interesting. The vitamin-A content of Ilish, Chital, and one of the

PLATE XXXIII.
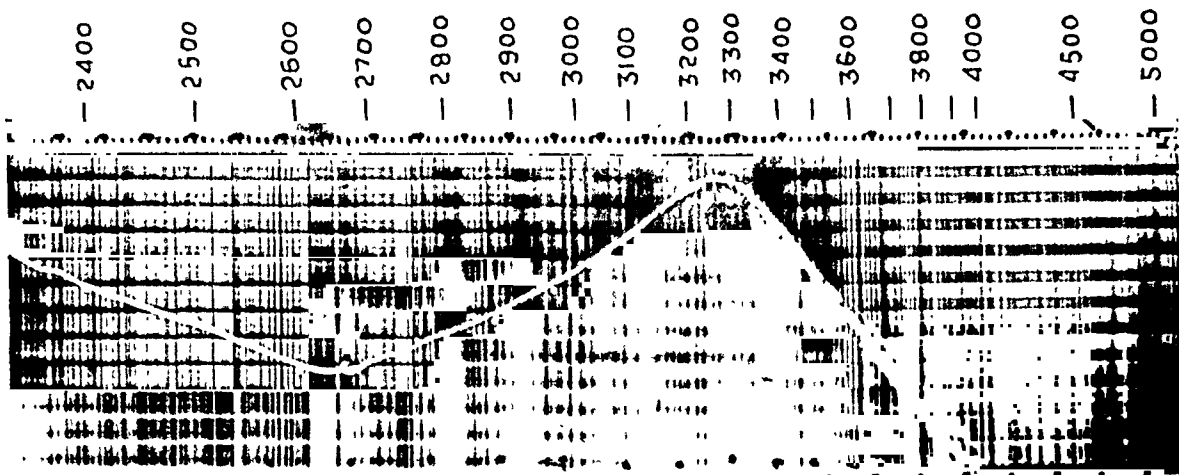
Halibut-liver oil.
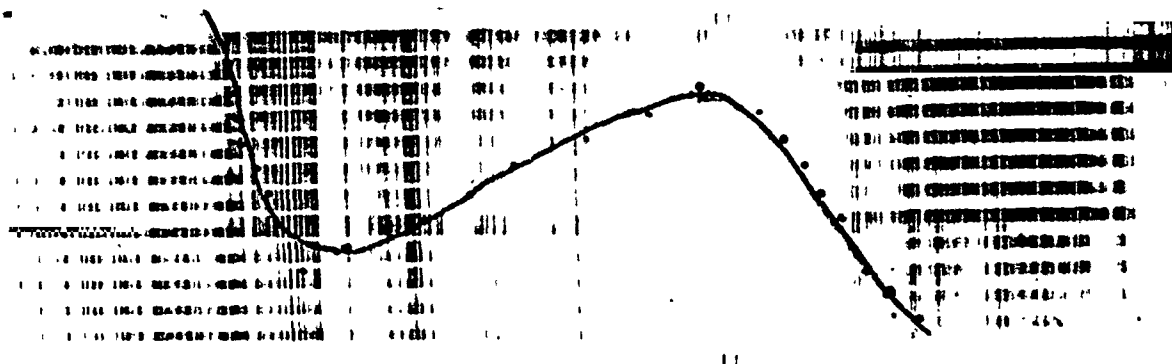
Kalbôs-liver oil.
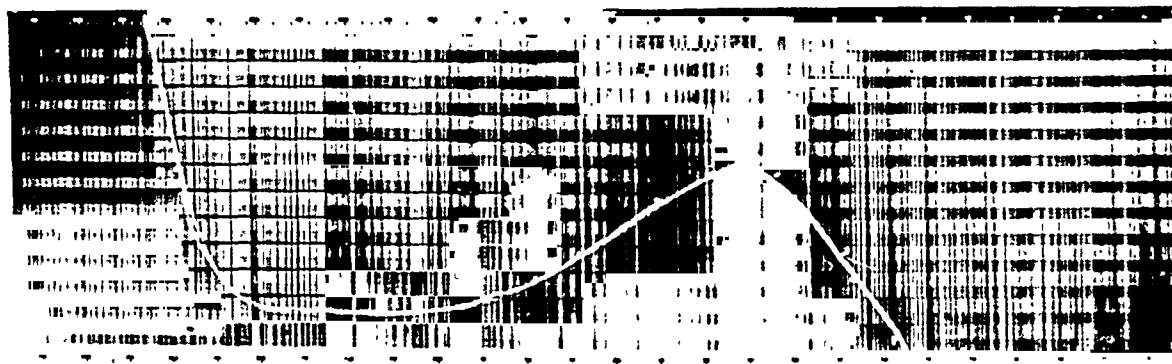
Rôhit-liver oil.
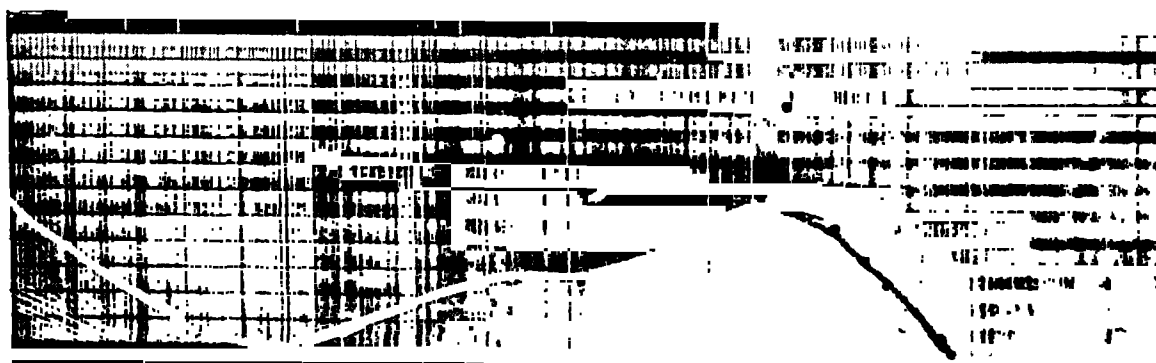
Arh-liver oil.

PLATE XXXIV.
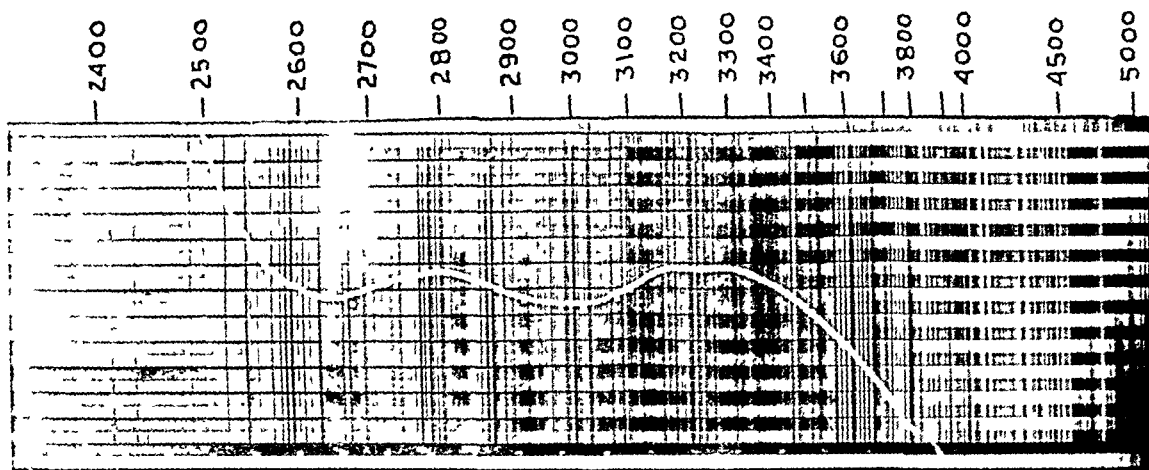
Shark-liver oil.
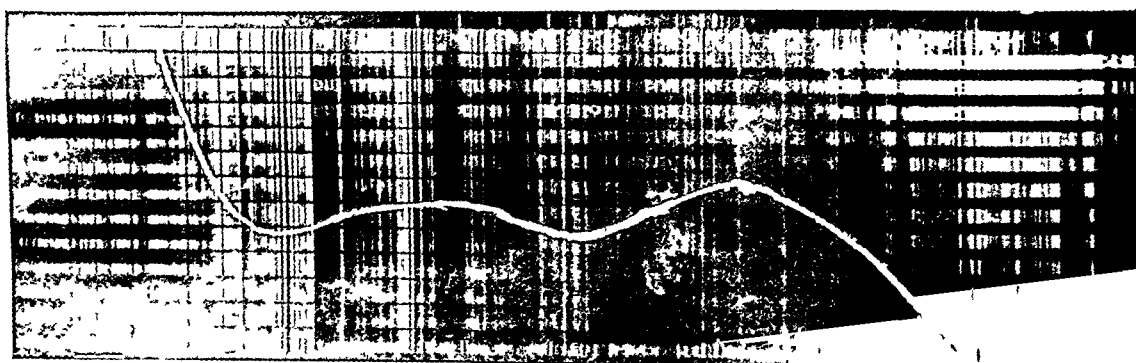
Ilish-liver oil.
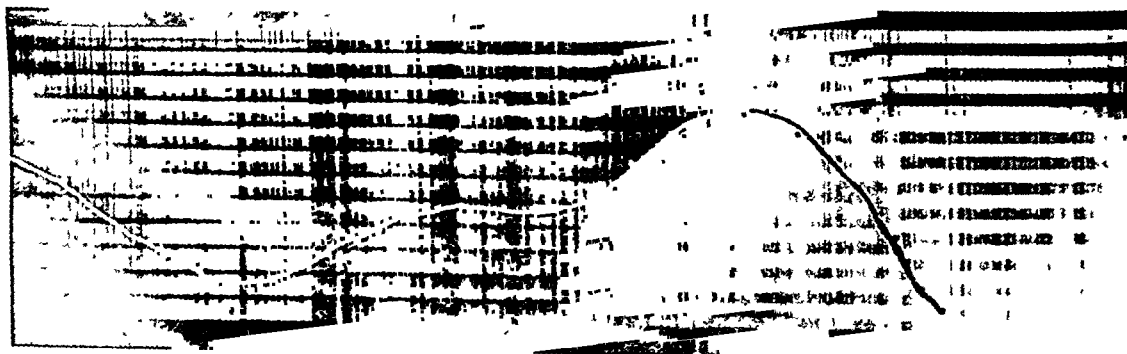
Bôal-liver oil.
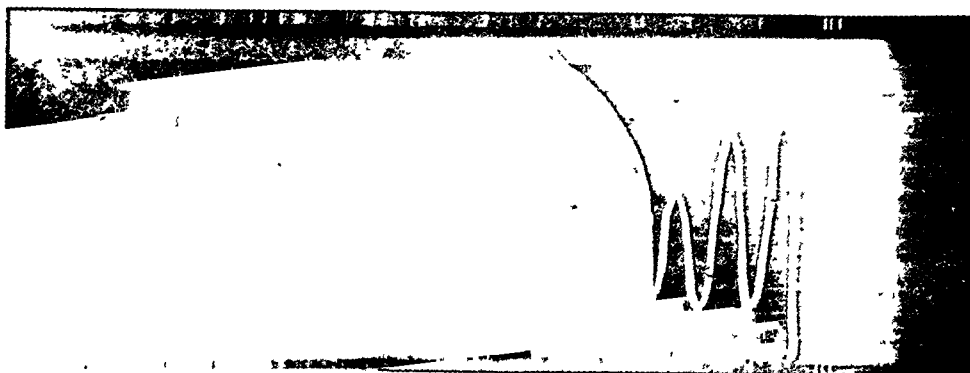
Bat-ray-liver oil (carotene).

sharks was very poor. All these fish were spawning at the time of the catch. The low fat content and vitamin-A content indicate that all the food and nutritive reserves have been utilized by the fish during the spawning season. Shorland (1938) has also observed the same phenomena while studying the vitamin-A content of New Zealand fish. In the other cases, particularly the carps, the fat and vitamin-A content were higher in the month of March than in November. The spawning season of these fish was in July and hence the March catch was more mature than the November ones. These observations show that once the fish has come to an adult stage there is no considerable fluctuation in their store of vitamin A. Particular mention must be made of the low vitamin-A content of Ilish. All the fish caught and brought to the market are young, the adult ones having gone to the sea. So the low vitamin-A content may be due to both the season and maturity.

In some cases, as Dhain, bat-ray, Shillang. and Shôle, the liver oils contained a large amount of carotene, while the deposit fats were colourless or light yellow. This shows that carotene also is stored only in the liver though it is fat-soluble. The observation of both carotene and vitamin A at the same time in the liver oils give considerable support to the hypothesis that the liver is probably the seat of conversion of carotene into vitamin A.

The relation between the Carr-Price value and the E value has been remarkable. For practically all the rich liver oils, i.e., whose E value was higher than 3 and Carr-Price value higher than 100, the Carr-Price value was lying between 25 and 30 times that of the E value. Even though considerable precaution has been taken for the accurate measurement of the blue units, we cannot expect a better agreement considering the transient nature of the blue colour and the unsatisfactory method of matching by tinted glasses (*vide* Chevalier and Chambre, 1933). This observation also proves that the blue colour and the absorption band at $3280\overset{\circ}{A}$ is definitely due to vitamin A. One Lovibond blue unit of Carr-Price test works out to be roughly 55 International Units per gramme of vitamin A.

SUMMARY.

The liver oils of 16 different species of fish, both fresh-water and marine, are rich sources of vitamin A, some containing 30 times the vitamin-A content of average cod-liver oil and nearly $\frac{3}{4}$ that of halibut-liver oil. Six species gave from 10 to 20 times and 6 from 5 to 10 times the vitamin-A content of cod-liver oil.

During the spawning season the vitamin-A content of the liver oils is poor, while in the growth season they are fairly rich.

Most of the fresh-water fish-liver oils showed an absorption spectrum with the maximum of the vitamin-A band shifted to the long wave-length region at about $3450\overset{\circ}{A}$ with an additional inflexion or a band at $2900\overset{\circ}{A}$. The shark, cod, and halibut-liver oils showed an absorption band with a maximum at $3280\overset{\circ}{A}$.

The preparation of the liver oils by steaming or cooking in water in contact with air caused 20 to 60 per cent loss of vitamin A.

The oils prepared from the fats deposited around the liver do not contain appreciable amounts of vitamin A or carotene.

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VITAMIN-A CONTENT OF SOME SPECIES OF BENGAL FISH BY BIOLOGICAL, TINTOMETRIC, AND SPECTROSCOPIC METHODS.

BY

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(*An Inquiry under the Indian Research Fund Association.*)

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IN a previous communication from this laboratory Basu and De (1938) determined the vitamin-A contents of the body and liver oils of *Labeo rohita* and *Clupea ilisa* by the biological method. The vitamin-A contents of the body and liver oils of several other common species of Bengal fish have been determined by three methods, viz., biological, tintometric, and spectroscopic, and the results obtained form the subject-matter of this communication.

EXPERIMENTAL.

Preparation of the oil.—The livers of Ruhee (*Labeo rohita*), Katal (*Catla catla*), Mrigel (*Cirrhina mrigala*), Air (*Arius arius*), Hilsa (*Clupea ilisa*), Naindal, and Sarputi (*Barburs sarana*) were collected during the months of August and September. The finely minced liver was ground up with excess of anhydrous sodium sulphate and extracted in a soxhlet apparatus with ether. The ether was distilled off and finally the oils were dried.

For the extraction of the body oils the fishes Hilsa (*Clupea ilisa*), Chital (*Notopterus chitala*), Kôi (*Anabus testudineus*), and Magur were collected during the early

part of October. The fish meal was heated in a porcelain basin on the water-bath. The separated oils were extracted with ether repeatedly in the cold. The ether was evaporated and the oil dried.

Generally the oils had a colour, varying from deep yellow to yellowish brown. They had their peculiar odours. Oils were preserved in an atmosphere of nitrogen in the refrigerator.

TECHNIQUE.

(a) *Biological method.*—The technique employed was the rat-growth method as in the previous investigation of Basu and De (*loc. cit.*).

(b) *Tintometric method.*—For the oils themselves the technique of Carr and Price (1926) was followed and the blue colour was matched against standard glasses in a Lovibond tintometer. The oils which were rich sources of vitamin A were diluted 2 to 4 times, as was necessary, before the experiment so that Lovibond blue value might be obtained within the range of 6 to 8 units. It was observed that the relation between dilution up to this range and the intensity of colour produced was approximately linear. The relation will not be linear at great dilutions as was observed by Chakravorty, Mukherjee and Guha (1933).

The tintometric method was also applied to the unsaponifiable fractions of the oils. The oils were saponified by the method of Smith and Hazley (1931) and the unsaponifiable portion from 2 g. of an oil was dissolved in 10 c.c. of chloroform and the solution thus prepared was equivalent to 20 per cent solution of the original oil. The subsequent procedure was the same as was adopted with the oils.

(c) *Spectroscopic method.*—The apparatus used was an Adam Hilger E_3 Quartz spectrograph. The light source was a condensed spark between the two electrodes of iron-tungsten alloy, and a rotatory sector photometer was placed between the slit and the source of light.

The oil was dissolved in absolute alcohol and the concentration of each oil is given in Graphs. 1 and 2. The length of the absorption tube was 2 cm. The spectrophotograph for each oil solution was taken for several density readings on extra thin Ilford special rapid plates. The points at which equal intensities of lines in the upper and lower spectra occurred were joined to give the absorption curve. This is represented graphically with wave-length as abscissa and log I_0/I as the ordinate in Graphs 1 and 2. Wave-length was measured by comparing the positions of special lines with lines in standard chart and log I_0/I was obtained by dividing the density readings on the sector by the length of the absorbing tube through which light passed.

RESULTS.

(a) *Biological method.*—The growth responses in young rats for different doses of oil (both liver and body) are summarized in Table I.

TABLE I.

Growth response of rats in 28 days with various doses of vitamin A containing supplements and the vitamin-A contents of oils.

Name of the supplement.	Number of rats used.	Dose.	Increase in weight per rat.	Increase in weight per rat per week.	Vitamin A units per gramme of oil.
Carotene	7	4 units	13·5 g.	3·4 g.	..
., ...	6	4 ,,	14·0 ,,	3·5 ,,	..
., ...	5	4 ,,	15·0 ,,	3·8 ,,	..
Katal-liver oil ..	8	10 mg.	8·0 ,,	2·0 ..	} 207
,, ,, ,, ..	7	20 ,,	14·0 ,,	3·5 ,,	
Mrigel-liver oil ..	8	10 ,,	12·7 ,,	3·2 ,,	377
Basal diet only ..	5	*Nil.*	*Nil.*	*Nil.*	..
Kôi-body oil ..	7	80 mg.	−6·1 g.	−1·6 g.	*Nil.*
Magur-body oil ..	6	80 ,,	−13·2 ,,	−3·2 ,,	*Nil.*
Air-liver oil ..	7	20 ,,	14·0 ,,	3·5 ,,	200
Basal diet only ..	4	*Nil.*	*Nil.*	*Nil.*	..
Naindal-liver oil ..	6	10 mg.	15·0 g.	3·7 g.	405
Sarputi-liver oil ..	4	20 ,,	−17·6 ,,	−4·4 ,,	*Nil.*
Chital-body oil ..	5	100 ,,	10·0 ,,	2·5 ,,	26
Basal diet only ..	4	*Nil.*	*Nil.*	*Nil.*	..

(b) *Tintometric method.*—The results are indicated in Table II which represents the average of several consecutive readings :—

TABLE II.

Amounts of blue units (expressed as Carr-Price value) with oils as such and with the unsaponifiable portion of the oil. The average only of several consecutive readings are given.

Nature of the oil.	WITH OIL AS SUCH.			WITH UNSAPONIFIABLE PORTION OF THE OIL.		
	Amount of yellow units.	Amount of red units.	Carr-Price value.	Amount of yellow units.	Amount of red units.	Carr-Price value.
Cod-liver oil (B. C. P. W.).	4·0	..	6·0	4·5	..	7·5
Ruhee-liver oil ..	2·0	..	7·0	6·0	..	14·0
Katal-liver oil ..	1·0	..	2·5	2·0	..	2·5
Kôi-body oil ..	..	..	Positive	1·0	..	1·2
Hilsa-liver oil ..	..	..	,,	4·0	..	1·5
Mrigel-liver oil ..	2·0	..	7·5	6·0	..	12·5
Magur-body oil ..	..	..	Trace	1·0	..	2·6
Air-liver oil ..	1·5	..	4·75	2·0	..	4·6
Chital-body oil ..	2·0	1·0	1·25	2·0	1·0	2·9
Naindal-liver oil ..	2·0	..	12·0	6·0	..	20·2
Sarputi-liver oil ..	..	..	2·0	1·5	..	3·0

(c) *Spectroscopic method.*—The absorption spectra of the oils are represented graphically in Graphs 1 and 2 :—

GRAPH 1.
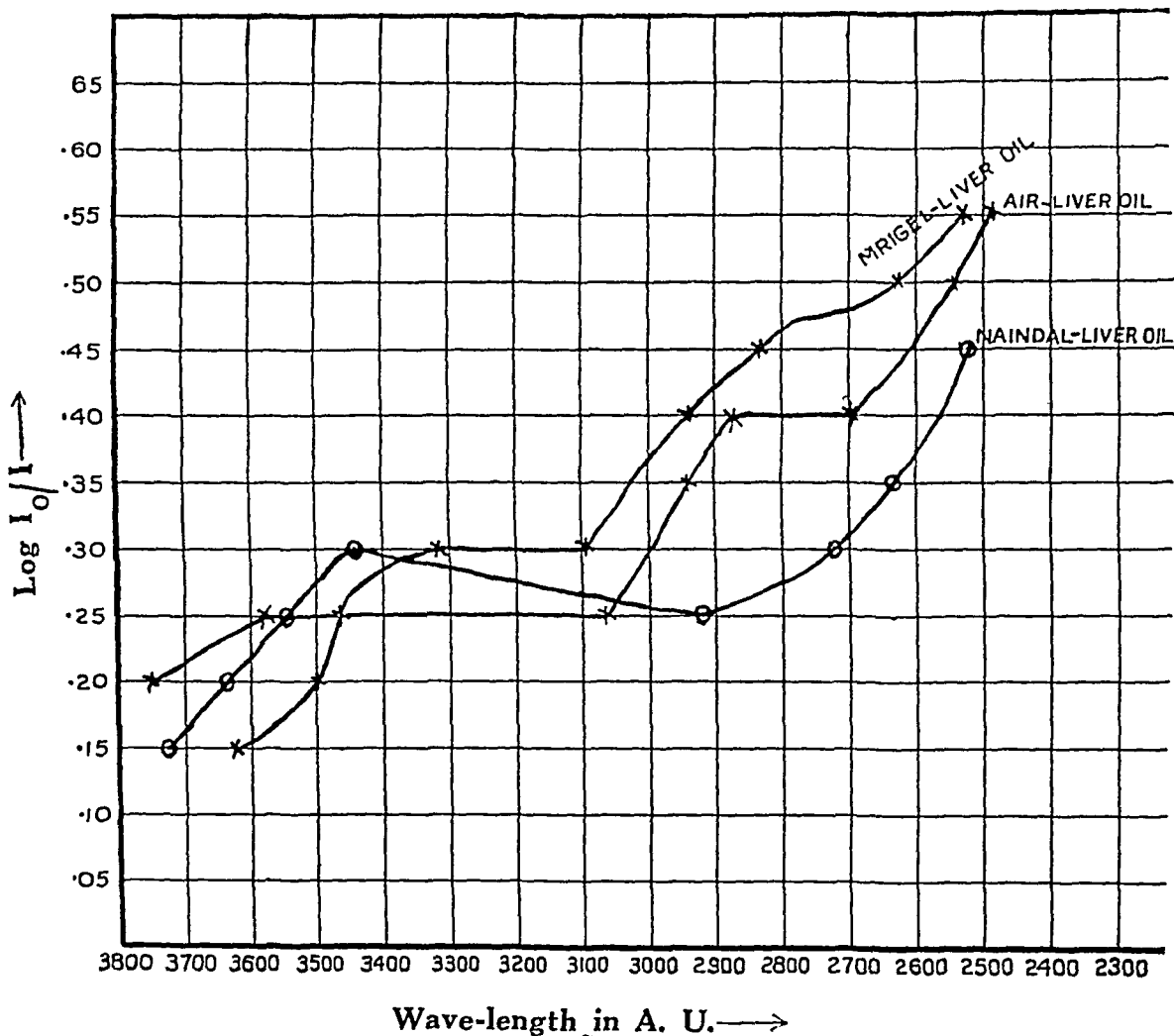
Ruhee- and Naindal-liver oils exhibit clear absorption bands at 320μ to 330μ. Hilsa-body oil exhibits general absorption and no selective absorption is detected, showing that the vitamin-A content is negligible. In the case of oils of other fishes no fine structure at 328μ is detectable. So quantitative study fails. Although

the curves show mostly general absorption, there are two flat portions in the absorption curves of Mrigel-liver oil, Air-liver oil, and Chital-body oil, in the regions 350μ to 310μ and 260μ to 290μ, respectively. Katal-liver oil shows a long band from

GRAPH 2.

Wave-length in A. U.——→
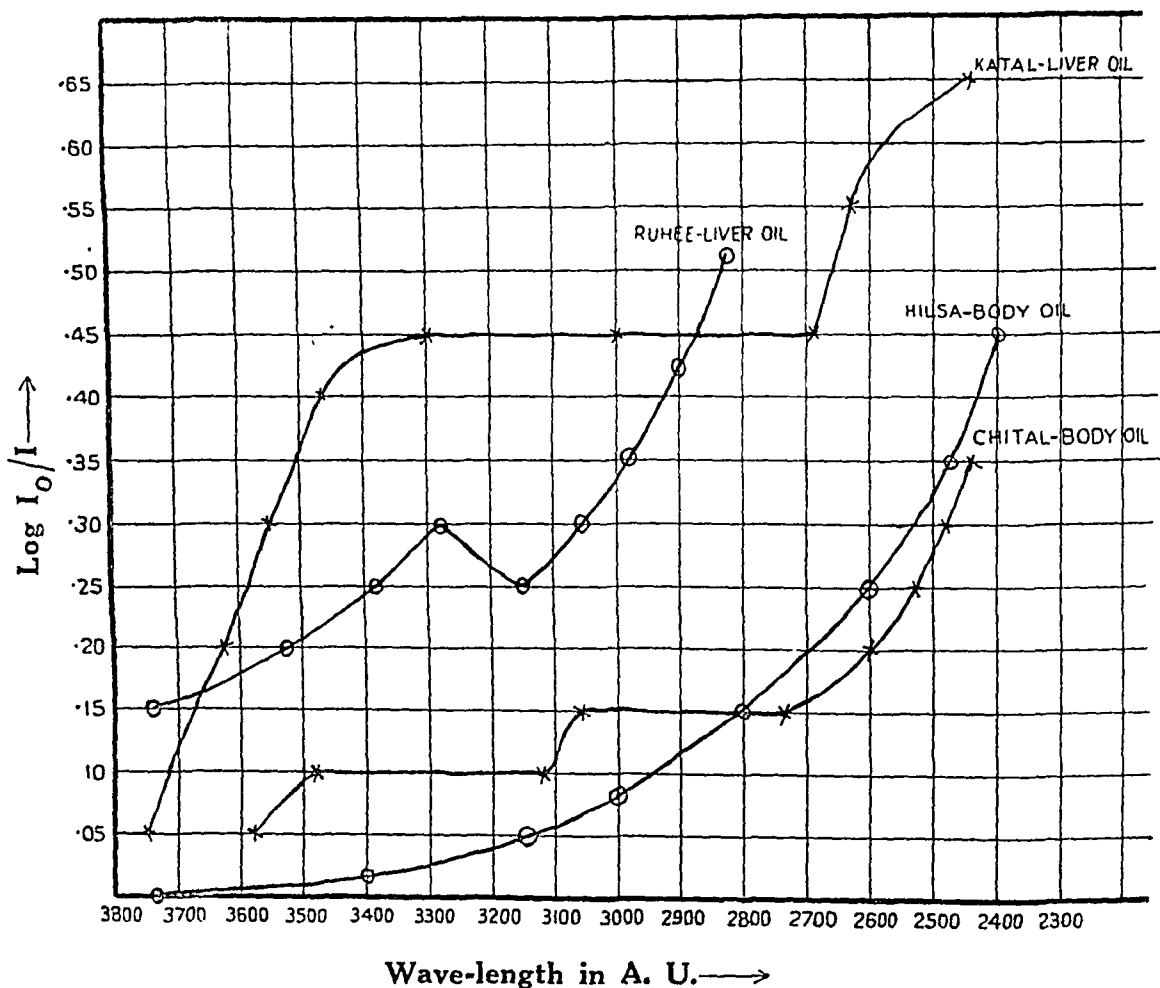
340μ to 270μ. With none of the oils an absorption band at 345μ to 350μ characteristic of vitamin A_2, which was observed by Gillam *et al.* (1938), and Lederer and Rathmann (1938) in Russian fresh-water fish, could be detected.

Method of calculation.—Method of calculating the vitamin-A content from absorption spectra may be indicated in the case of Naindal-liver oil.

A concentrate of *Naindal-liver oil* containing 0·005 g. of the oil per c.c. showed a value for log I_0/I of 0·35 when photographed through 2 cm. cell. The value for the extinction co-efficient of 1 g. of oil at 328μ will, therefore, be equal to $\left(\frac{0·35}{2} \div 0·005\right)$, where 2 equals the depth of the cell in cm. and 0·005 equals the g. of the oil per c.c. of the solution photographed.

Therefore E the extinction co-efficient (1 g. at a depth of 1 cm.) = 35 at 325μ. The vitamin-A values are calculated according to the Beer-Lambert's Law,

$$E = \frac{1}{c \times d} \log I_0/I,$$ when C = conc. of substance, d = length in cm. of liquid column through which light passes, I_0 = intensity of incident light and I = intensity of transmitted light.

If the extinction co-efficient for pure vitamin A be taken as equal to 1,600* (Carr and Jewell, 1933) and substituted in the above formula.

$C = \frac{0·35}{1,600 \times 2} = 1·09$ vitamin A per c.c. of the solution.

i.e., 0·005 gm. of oil ≡ 1·09 γ vitamin A

or 1 gm. of oil ≡ 218 γ vitamin A

≡ 727 rat units of vitamin A.

Table III shows the vitamin-A content of the two oils by the spectrographic method. Other oils did not give sharp absorption band at 328μ.

TABLE III.

Nature of the oil.	Vitamin-A value per g. of the oil, International Units.	Weight of the oil (g.) containing 1 I. U. of vitamin A.
1. Naindal-liver oil ..	727 I. U.	0·0013
2. Ruhee-liver oil ..	622 I. U.	0·0016

*Holmes and Corbet (1937) have successfully prepared crystalline vitamin A from the unsaponifiable fraction of the liver oil of *Stereolepis ishinagi* by low temperature crystallization from organic solvents containing a very small amount of water. Spectroscopic examination gives a value for E 1 per cent, 328μ of 2,000 compared with the accepted value of 1 cm. 1,600.

DISCUSSION.

In Table IV the vitamin-A content of the body and liver oils of different species of Bengal fish determined by different methods is shown :—

TABLE IV.

The relative vitamin-A values of fish liver and body oils obtained by three different methods.

Nature of the oil.	Zoological name of the fish.	Carr-Price value with oil as such.	Carr-Price value with unsaponifiable portion.	Units vitamin A per g. of the oil (biological method).	Units vitamin A per g. of the oil (spectroscopic method).
1. Ruhee-liver oil ..	*Labeo rohita*	7·00	14·00	461 (Basu and De)	622
2. Naindal-liver oil..	....	12·00	20·20	405	727
3. Katal-liver oil ..	*Catla catla*	2·50	2·50	207	..
4. Air-liver oil ..	*Arius arius*	4·75	4·60	200	..
5. Mrigel-liver oil ..	*Cirrhina mrigala*	7·50	12·50	377	..
6. Sarputi-liver oil ..	*Barbus sarana*	2·00	3·00	*Nil*	..
7. Hilsa-liver oil ..	*Clupea ilisa*	Trace	1·50	199 (Basu and De)	..
8. Chital-body oil ..	*Notopterus chitala*	1·25	2·90	26	..
9. Hilsa-body oil ..	*Clupea ilisa*	..	..	*Nil* (Basu and De)	..
10. Magur-body oil ..	....	Trace	2·60	..	..
11. Koi-body oil ..	*Anabus testudineus*	,,	1·20	?	..
12. Cod-liver oil (B. C. P. W.)	....	6·00	7·50	195–200 (Datta and Banerjee)	..
13. Halibut-liver oil ..	....	(Parke, Davis & Co.)	..	55,000	..
14. Cod-liver oil ..	....	(International Standard)	..	1,000	..

It will be observed that body oils of fish contain little or no vitamin A. The liver oils of Ruhee, Naindal, Katal, Air, Mrigel, and Hilsa, contain appreciable amount of vitamin A as measured by the biological method. The Ruhee-, Naindal-, Mrigel-, and Katal-liver oils are very good sources of vitamin A and

indeed contain more vitamin A than the imported varieties of cod-liver oil which lose much of their potency in transit and on storage. The Naindal- and Ruhee-liver oils are potent sources of vitamin A, the former containing 727 and the latter 622 International Units per g. as measured by the spectroscopic method; these two oils, therefore, almost reach the standard of cod-liver oil. Mrigel-, Katal-, and to a less extent Air-liver oils also, contain appreciable amounts of vitamin A.

It is remarkable that none of the liver oils of fresh-water Bengal fish showed an absorption band at 345μ to 350μ characteristic of vitamin A_2.

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STUDIES ON VITAMIN-A DEFICIENCY.

Part IV.

THE EFFECT OF CAROTENE ON THE PERIPHERAL NERVE LESIONS PRODUCED BY VITAMIN-A DEFICIENCY.

BY

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DEGENERATION of varying degrees in the myelin sheaths of the spinal nerves produced in young animals by diets deficient in vitamin A and carotene has been previously described (Radhakrishna Rao, 1938a). It was reported that while histological examination of the peripheral nerves revealed myelin degeneration in most of the animals fed on the deficient diets, clinical evidences of nervous disorder, such as general inco-ordination, sluggish movement or ' leg weakness ' were rarely observed. Similar changes were also described in the trigeminal and optic nerves (Radhakrishna Rao, 1936). It was found that the addition of adequate amounts of carotene to the deficient diet, after the animals had developed typical signs of xerophthalmia, resulted in recovery of the eye condition but that the myelin degeneration persisted in the trigeminal nerve. The period of treatment with carotene was, however, short in this experiment, which was devised for a different purpose, i.e., to find out the relation between the epithelial and nervous lesions occurring in vitamin-A deficiency. Hence further work was undertaken to investigate the effect of the prolonged administration of carotene on the peripheral nerve lesions. In the present investigation the spinal nerves were chosen for study.

EXPERIMENTAL.

Twelve young rabbits, which had been fed on a good mixed diet from birth, were placed in separate cages and given the following diet, deficient in carotene and vitamin A :—

				Parts.
Crushed oats	..	..	..	68·5
Rice bran	..	..	..	20·0
Calcium carbonate	..	..	..	1·5
White potatoes	..	..	..	10·0

Calcium carbonate was added to provide enough calcium, as it has been shown that nervous lesions can be produced in animals by diets rich in cereals but containing very little available calcium (Stockman, 1934; Stockman and Johnston, 1933; both quoted by Mellanby, 1935). White potatoes were added to the diet to supply vitamin C; their carotene content is negligible. The animals were weighed weekly and an examination of the eye was made at frequent intervals. Soon after the animals had developed typical manifestations of xerophthalmia, 10 grammes daily of fresh green amaranth leaves (*Amaranthus gangeticus*), supplying roughly 250 micrograms of carotene, were added as a supplement. In one case (rabbit No. 5) 0·5 ml. of red-palm oil (*Elaeis guineensis*) daily was substituted for the amaranth leaves; one ml. of red-palm oil supplies about 410 micrograms of carotene.

The animals were examined every day after the institution of the above treatment and the time taken for the disappearance of the eye signs noted. Rabbit No. 1 was killed soon after the xerophthalmia had cleared up and the changes in the peripheral nerves after this short period of treatment investigated. The other animals were killed at various intervals after the commencement of treatment with carotene (*see* Table) but at definite intervals (1 to 14 weeks) after the disappearance of the xerophthalmia.

To avoid post-mortem changes, the animals were killed by air embolism and some of the peripheral nerves (sciatic and femoral nerves, nerves from the brachial plexuses) were removed soon after death. Immediately after removal the nerves were fixed in Müller's fluid and 10 per cent neutral formalin. Preparations for histopathological study were made from the material fixed in Müller's fluid by Marchi's method. Frozen sections of the nerves fixed in neutral formalin were stained by Scharlach R and Sudan IV. These sections were also examined in polarized light between crossed Nicol prisms. The advantages of this method in the study of myelin degeneration were pointed out in a previous communication (Radhakrishna Rao, 1938b).

Observations.

Though there were variations in the time of onset of xerophthalmia, all the animals, with the exception of two, showed about the same degree of the eye condition when the treatment with carotene was commenced. In these two animals (Nos. 3 and 7), the development of the ocular signs was so sudden and marked that corneal ulcers had actually developed before the treatment was started. In both these animals, the corneal ulcers took about 6 weeks to heal and permanent opacities of the cornea, which impaired the vision, remained as sequelæ. In all the other animals, the xerophthalmia cleared up under treatment in less than two weeks without leaving any trace on the cornea.

All the animals gained in weight after the addition of carotene to the deficient diet. In 3 animals, however, the response was not very marked. At the end of the experiment, it was found that the livers of all the animals were positive to the antimony-trichloride test for vitamin A.

A moderate degree of myelin degeneration was found in the nerves of rabbit No. 1, which was killed soon after the xerophthalmia was cured. Examination of the peripheral nerves from the remaining rabbits revealed that degeneration of the

medullary sheaths was still present in spite of the clinical improvement. In sections stained by Marchi's method, most of the degenerated fibres in rabbit No. 1 showed the ' annular ' type of degeneration ; fine or coarse granules of disintegrated myelin, stained black by osmic acid, were mostly seen in sections of nerves removed from the other animals. The duration of treatment with carotene has had no appreciable effect on the peripheral nerve lesions. Slight but definite de-myelination of nerve-bundles in the peripheral nerves was still present even in animals killed 12 and 14 weeks after the disappearance of xerophthalmia. Study of the frozen sections of the nerves stained by Scharlach R and Sudan IV confirmed the above findings. Irregular swelling, fragmentation, and the presence of isotropic material in the myelin sheaths of the nerves examined in polarized light and between crossed Nicols conclusively showed that slight myelin degeneration was present in all the animals irrespective of the period of treatment.

Rabbit No. 5 treated with red-palm oil also showed similar changes in the peripheral nerves.

TABLE.

Effect of carotene treatment on peripheral nerve lesions.

Animal number.	Condition of the eye.*		Treatment (time in weeks after disappearance of xerophthalmia).	Weight (grammes).			Lesions of the peripheral nerves (microscopic diagnosis).†				
	Right.	Left.		Initial.	Before treatment.	Terminal.	Sciatic.	Femoral.	Median.	Ulnar.	Radial.
1	+	+	0 (Killed soon after disappearance of xerophthalmia.)	1,425	1,265	1,300	2	1	2	2	2
2	+	+	1	1,225	1,260	1,325	1	1	1	1	2
3	++	+	2	1,545	1,275	1,560	1	1	1	1	1
4	+	+	3	1,040	1,390	1,375	1	1	1	1	1
5	+	+	4	905	1,520	1,815	1	1	1	1	1
6	+	+	5	1,400	1,360	1,480	1	1	1	1	1
7	+	++	6	1,435	1,695	1,545	1	1	1	1	1
8	+	+	7	1,060	1,115	1,375	1	1	1	1	1
9	+	+	8	1,440	1,630	1,615	2	1	1	1	1
10	+	+	10	1,230	1,100	1,340	2	1	1	1	1
11	+	+	12	1,310	1,420	1,500	1	1	1	1	1
12	+	+	14	1,565	1,635	1,840	1	1	1	1	1

* + = Xerophthalmia.
++ = Corneal ulcer.
† 0 = No degeneration.
1 = Slight degeneration.
2 = Moderate degeneration.
3 = Marked degeneration.

DISCUSSION.

Several workers have described neurological manifestations in experimental animals fed on vitamin-A deficient diets and showed that carotene, when added to the deficient diets from the beginning of the experiment, was effective in preventing the nervous lesions. Sutton, Setterfield and Krauss (1934) found that the early degenerative lesions in the sciatic and femoral nerves at the time of incipient ophthalmia were unhealed in rats which had received adequate amounts of vitamin A for a period of 8 weeks after the appearance of the eye lesions. Zimmerman and Cowgill (1936) similarly found that in vitamin-A deficient rats given adequate treatment with carotene (4 drops of a 0·1 per cent solution in cotton-seed oil, equivalent to 100 micrograms, a day) over a period ranging from 4 to 31 days, the neurologic manifestations remained unimproved throughout the course. In the present investigation the effect of carotene on nerve degeneration has been more systematically studied, with results which confirm and extend those of the above workers.

In the present experiments, it was found that the administration of carotene was effective in improving the clinical condition and in arresting the further progress of degeneration in the peripheral nerves, but was ineffective in curing the lesions already present before treatment. It appears that the nerve lesions of the kind noted persist for a long time and administration of carotene at a late stage in the deficiency has no demonstrable effect on such lesions. It is also clear that minor degrees of de-myelination of the nerves may be present without any clinical evidence of nervous disorder.

Varying degrees of peripheral neuritis are commonly met with in South Indians. It is generally believed that the condition is due mainly to deficiency of vitamin B_1. The possible rôle of vitamin-A deficiency in producing this condition has not, however, received adequate attention. Judging from the prevalence of keratomalacia and xerophthalmia in South India, vitamin-A deficiency appears to be a common condition in the poorer classes and it may perhaps play a part in the production of peripheral neuritis.

SUMMARY.

A study has been made on rabbits of the effect of adding adequate amounts of carotene to a vitamin-A deficient diet, after the animals had developed xerophthalmia. While recovery from xerophthalmia took place, anatomic lesions in the peripheral nervous system persisted and showed no evidence of regeneration.

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PIGMENTATION OF THE CONJUNCTIVA AND ITS POSSIBLE RELATION TO NUTRITION.

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PIGMENTATION of the conjunctiva has been the subject of a fair amount of observation all over the world. The efforts of some of the workers have been directed towards the discovery of some method of diagnosis of vitamin-A deficiency long before the appearance of definite clinical signs such as Bitot's spots and keratomalacia. Pillat (1933) studied sections of the conjunctiva of Chinese both in apparently good health and showing conditions indicative of vitamin deficiency, such as night-blindness, prexerosis, xerosis, and keratomalacia. He states that the ' normal ' conjunctiva in the Chinese contains a large amount of pigment not visible to the unaided eye, which may be due to the fact that the great majority of the Chinese live on the borderline of vitamin-A deficiency. Probably the same holds good in India judging from the diet surveys carried out in the provinces of Bombay, Madras, and Bengal. Pigments can accumulate in greater amounts in certain regions of the conjunctiva. Fisher (1905) reported accumulation of pigment in the epithelial cells of the conjunctiva at the limbus on microscopic examination in various tribes in Africa. Kazao (1919) in Japan described a similar condition in Japanese among whom the pigmentation was visible to the unaided eye. Wright (1922) in India, and Mori (1924) have recorded their observations on keratomalacia and xerosis epithelialis conjunctivæ, respectively. But so far, pigmentation of the conjunctiva similar to that which has been observed among the poor population of Bombay has not been described.

In the course of a routine medical examination conducted in an industrial school in Bombay, it was found that the conjunctiva of a great number of boys was definitely pigmented. Practically all the inmates of this school came from the slums of the city and were convicted by the juvenile court for vagrancy and petty offences and handed over to the care of the industrial school. The ages of the boys examined ranged from 11 to 16 years. The pigment deposited in the conjunctiva

was in the form of (*a*) elliptical or irregular patches and (*b*) rings, semi-circles or segments round the cornea. The patches of pigment are light brown in colour and are for the most part situated on the outer side of the exposed portion of the conjunctiva midway between the cornea and the outer canthus. The margins are ill defined and merge insensibly into the surrounding conjunctiva which is dirty white, lustreless, and unhealthy in appearance. At times fine blood-vessels may be seen in the area suggesting chronic congestion. These patches in the majority of cases exist in both eyes. The linear pigmentation is dark brown in colour and is situated at the sclerocorneal junction or at a very short distance away from it into the scleral region. It is 0·5 to 2·0 millimetres wide, has well-defined margins, and curves round the cornea as segments, semi-circles, or as complete circles. In some cases it is found at or near the lower margin of the cornea in the form of a curved line, the central portion being near the cornea, while the extremities are carried further away from it into the sclera on both the sides of the cornea. At times two such lines exist, one at the upper and the other at the lower margin of the cornea forming an ellipse, the long axis coinciding with the long axis of the rima palpabrum, and in this way enclosing a good portion of the bulbar conjunctiva. This linear pigmentation is observed as a rule in both the eyes.

On examination, the conjunctivæ of 169 out of 189 boys were found to be pigmented. On account of the high incidence of pigmented conjunctivæ, it was suspected that the destitute condition of the boys and all that this connotes, especially with regard to their diet, might be a causal factor in the production of the pigment. Hence, for purposes of comparison, an inspection of children belonging to a higher social class and attending a day school wherein the fees are higher than the average tuition fees of schools in Bombay, was carried out. Free students were excluded from examination. One hundred and sixty-four children of about the same ages as the boys in the industrial school were examined ; 44 of them showed brown pigment in the conjunctiva. The fact that all the children examined were paying students does not necessarily mean that they were adequately nourished or were properly looked after by their parents as is evident from the fact that one child was suffering from a definite Bitot's spot in each eye. However, the low incidence of pigmented conjunctiva among children in the day school is in contrast to its prevalence among the boys in the industrial school.

TABLE I.

Incidence of conjunctival pigmentation among children of the day and industrial schools.

School.	Sex.	Number examined.	Linear pigmentation.	Patchy pigmentation.	Percentage of children with pigment.
Day school ..	Boys	115	32	0	27·8
	Girls	49	12	0	24·7
Industrial school ..	Boys	189	143	26	89·4

Since a number of boys of the industrial school were found to have defective vision, it was felt desirable to know whether this condition had any relation with the conjunctival pigmentation observed by us. For this purpose Snellen's test at a distance of 20 feet was carried out. It was observed that, out of 189 boys examined, 137 had defective vision in one or both eyes. The degree of bad vision varied from 6/9 to even as much as 6/60. As stated above, 169 boys had pigment in the conjunctiva out of which 119 boys had defective vision in one or both eyes. The incidence and character of pigment with or without defective vision are as follows :—

TABLE II.

Character of pigmentation and its distribution among boys with normal and defective vision.

Character of pigmentation.	Total number.	NORMAL VISION.		DEFECTIVE VISION.		Percentage with defective vision.
		Number.	Percentage.	One eye.	Both eyes.	
I. Boys without pigment.	20	1	5·0	1	18	95·0
II. Boys with conjunctival pigment.	169	51	30·2	18	100	69·8
A. Pigmented patches.	26	13	50·0	2	11	50·0
B. Linear pigmentation.	143	38	26·57	16	89	73·43
(*i*) Partial rings ..	85	20	23·41	12	53	76·59
(*ii*) Complete rings	58	18	31·03	4	36	68·97

The boys with defective vision were investigated further. Those with a vision 6/9, 6/9 or 6/6, 6/9 were left out of consideration as these were near the limit of normality. The pupils of boys having a vision 6/12 and less (one or both eyes) were dilated with atropine and an ophthalmoscopic examination for refraction and the condition of the retina was carried out. Those boys who had haziness or opacity of the cornea likely to interfere with vision, as also those who were difficult to examine, were excluded from the test. Full correction for refraction according

to the ophthalmoscopic reading was made by means of lenses, and Snellen's test done again. The results are as follows :—

TABLE III.

Improvement in vision after correction of refraction.

	WITHOUT CONJUNCTIVAL PIGMENT.		WITH CONJUNCTIVAL PIGMENT.	
	Number.	Percentage.	Number.	Percentage.
Vision 6/12 and less (total 82 boys).	12	..	70	..
(a) Complete improvement of vision.	6	50·0	24	34·3
(b) Incomplete improvement of vision.	6	50·0	46	65·7

From the table it will be clear that 6 out of the 12 boys without pigment, and 24 out of 70 boys with pigment, responded completely to correction of refractive errors by means of glasses. The rest, 52 in number, 6 without pigment and 46 with pigment when subjected to the same treatment did not show complete improvement in sight.

The appearance of the retina did not show any marked departure from the normal, though in a few cases the optic disc was found to be pale.

The high incidence of pigmented conjunctiva among the boys of the industrial school and the close association of their destitute condition with a poor dietary drew our attention to the diet consumed by the boys of the industrial school. The diet which consisted mainly of cereals and pulses with only a little mutton, oil, and jaggery, was adequate in total proteins and calories but lacking in milk and milk products. The figures obtained on chemical analysis for proteins, fats, and carbohydrates, were 88·7 g., 31·7 g., and 586·0 g., respectively, yielding a total of 3,060 calories per day per boy. The amount of animal proteins and fats were 4·2 g. and 0·4 g., respectively. The only 'protective' foodstuff in the diet was fresh vegetables—10·5 oz. daily per boy.

As the diet of the boys was presumably deficient in fat-soluble vitamins and as a great majority of them had pigmentation associated with lustreless conjunctiva, it was decided to ascertain the prevalence of night-blindness among them. Only 51 boys could be tested for night-blindness. Those who had defective vision had it corrected by glasses prior to the test. The technique adopted was that followed by Jeams and Zentmire (1934). The instrument used for the purpose was an electrically illuminated Birch Hirschfeld photometer (Carl Zeiss). The illumination was controlled by means of a glass wedge marked with scale of opacities and by the iris diaphragm, its full aperture measuring 20 mm. in diameter. Two readings with the glass wedge and the diaphragm were taken, one immediately after switching

PLATE XXXV.

Fig. 1. Showing the patch of pigment (P) in the conjunctiva. The white area within the patch is Bitot's spot medial to which is the pigmented ring round the cornea.

Fig. 3. Showing a thicker ring of pigment (R) than in Fig. 2.
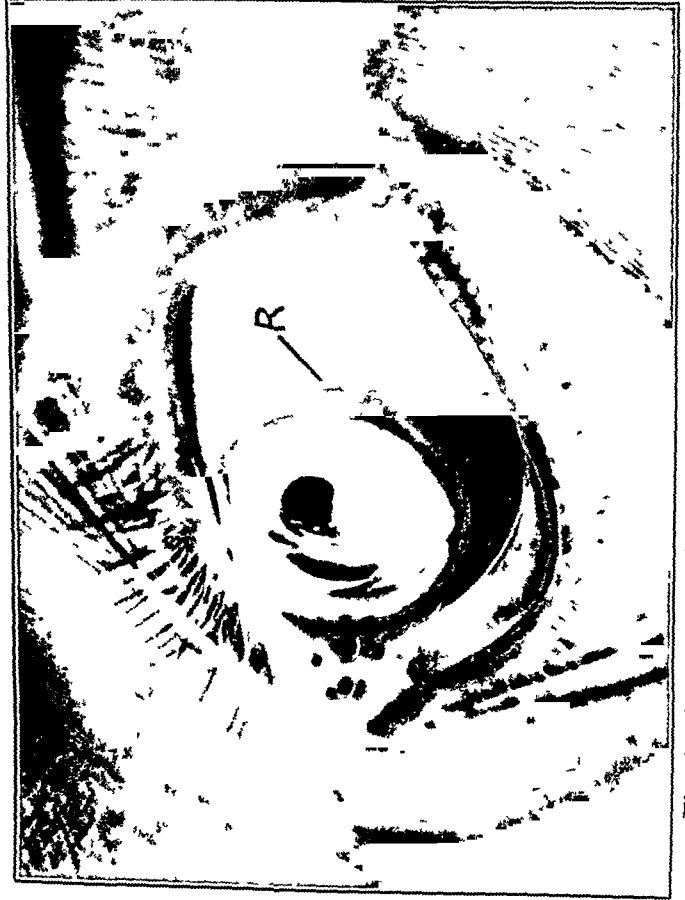
Fig. 2. Showing circular pigmentation (R) of the conjunctiva.

off a hundred-and-fifty Watt lamp to which the subject was previously exposed for five minutes, and the second at the end of ten minutes in total darkness. ' For the normal eye and with the wedge set at seven, the end-point opening of the iris diaphragm varied from 14 mm. to 20 mm. inclusive or if the wedge was set at six the reading was 8 to 10. During the ten minutes' period in darkness the normal eye recovered light sensitivity to the extent that with the wedge set at seven, the end-point opening of the iris diaphragm was from 3 mm. to 6 mm. inclusive. Wedge six was too transparent to be used for the normal eye after ten minutes in the dark ' (Jeams and Zentmire, *loc. cit.*).

Prior to subjecting the boys to the test, six members of the research and teaching staff of a medical college who presumably had normal vision and no vitamin-A deficiency were first tested with the instrument and the results were in agreement with the above quotation.

The results of tests on 51 boys are set out in Table IV :—

TABLE IV.

Incidence of night-blindness.

	Number of boys examined.	LIGHT SENSITIVITY.					
		NORMAL * (W7, D3 TO 6).		DIMINISHED W7, D8 TO 20.		GREATLY DIMINISHED W6 AND LESS D10 AND MORE.	
		Number.	Percentage.	Number.	Percentage.	Number.	Percentage.
1. Vision normal ..	34	15	44·12	18	52·94	1	2·94
(a) Conjunctiva without pigment.	9	8	88·89	1	11·11	*Nil*	..
(b) Conjunctiva pigmented.	25	7	28·00	17	68·00	1	4·00
2. Vision defective ..	17	3	17·65	6	47·06	8	35·29
(a) Conjunctiva without pigment.	2	*Nil*	..	1	..	1	..
(b) Conjunctiva pigmented.	15	3	20·00	5	33·33	7	46·67

* W = wedge.   D = diaphragm in mm.

Of boys with normal vision 55·88 per cent and of those with defective vision 82·35 per cent had diminished light sensitivity. Boys with conjunctival pigment suffered greatly from night-blindness, the incidence among them with normal and defective vision being 72 and 80 per cent, respectively. Though the incidence in both these groups is nearly the same, the boys with pigment but with defective vision were more advanced in night-blindness than those with pigment but with normal vision, as is evident from a comparison of the number of boys whose end-point in the test could not be reached at wedge seven and full diaphragm (20 mm.) after ten minutes in darkness. In the former group 46·67 per cent of boys had reached an advanced stage of night-blindness and in the latter only 4 per cent.

DISCUSSION.

It is well to review the possible causes of the appearance of the pigment. The pigmentary condition may be congenital. The fact that 89·4 per cent of children of one school have the pigmented conjunctiva, while only 26·8 per cent of those in another school suffer from a similar condition—both groups of children being of the same age and race—makes such a possibility improbable and remote.

Chronic conjunctivitis produced and maintained by prolonged exposure to strong sunlight and dust can form pigmentary patches in the conjunctiva. The boys of the industrial school working in the garden suffer from irritation and congestion of the conjunctiva for a few days only, due to the particles of earth lodging in the eyes. During the present investigations only one case of chronic conjunctivitis was detected. Pillat (*loc. cit.*) is of the opinion that work in strong sunlight is responsible for the increase in pigment which is evident only when the conjunctiva is viewed through the microscope; and that this increase is also dependent on certain dietetic factors responsible for creating hypersensitivity of the eyes with consequent photophobia. The routine diet of the school is singularly deficient in animal proteins and fats and probably also fat-soluble vitamins, and an average stay of three years in the institution may predispose to a condition of xerosis to a varying extent according to the state of health and nourishment prior to admission. This is supported by the fact that, though only few boys have been found to suffer from definite Bitot's spots, the great majority of children have conjunctivæ that are lustreless andtinted, on the whole appearing unhealthy, quite unlike that observed among the children of the higher middle class. This lack of lustre in the conjunctiva, which in the boys of the industrial school has been found to be present along with the pigmentation, may be associated with vitamin-A deficiency. When some of these boys were subjected to the photometric test for night-blindness it was found that the majority of boys who suffered from it were those with pigmented conjunctiva. A high percentage of boys whose vision did not completely improve after correction of refractive errors by glasses showed diminished sensitivity.

The existence of a possible relationship between the deposition of pigment in the conjunctiva in the form of patches and circles, and visual defects on the one hand and dietetic deficiencies on the other, require further elucidation with regard to the nature of pigment and its disappearance by administration of vitamin A.

SUMMARY.

1.   Out of 189 boys examined at an industrial school in Bombay 169 had patchy and linear pigmentation of the conjunctiva.   The diet of these boys was found to be extremely poor in animal proteins and fats.

2.   One hundred and thirty-seven had defective vision in one or both eyes, of these 118 had pigment in the conjunctiva as well.

3.   The vision of 52 boys did not return to normal after full correction of the refractive error by means of glasses.

4.   The percentage showing diminished light sensitivity was high in boys with pigmented conjunctiva.   Of those without pigmentation 88·89 per cent were normal, while only 28 per cent of those with pigment were normal as regards light sensitivity (Table IV).

Eighty per cent of boys with pigmented conjunctiva and defective vision which persisted even after correcting the refractive error showed impaired light sensitivity.

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PELLAGRA IN INDIA.

A STUDY OF TWENTY-FIVE CASES IN VIZAGAPATAM.

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CONTENTS.

I. INTRODUCTION.

PELLAGRA has a world-wide distribution. It is much more common in the temperate zone than in the Tropics. Although various authors have given priority to different people to the discovery of pellagra from 1578, the credit goes to Gasper Casal who described the dermal gastro-intestinal, and mental symptoms in 1735 in the Oviedo district in Spain, as one clinical entity and called the disease 'Mal de la Rose'. The word pellagra was introduced in 1771 by Frapolli, an Italian (*Pelle* = skin; *agra* = rough), the original name being 'PELLAGRA'.

Distribution of pellagra in India.—Megaw and Gupta (1927), writing about the distribution of diseases in India, reported four doubtful cases from Lyallpur, one from Calcutta, and one possible case from Natore. Since that date several cases have been reported in India. A summarized account of these has been given by Stannus (1936). Cases were reported in the following districts :—

Bengal : Megaw and Gupta (*loc. cit.*) reported a case definitely diagnosed as pellagra by Colonel Calvert from the Calcutta Medical College and one case reported by Colonel Sandes from Natore ; Panja (1935) reported two cases from Calcutta, both in Hindu widows, the first case seen by him was in 1933. Sen Gupta *et al.* (1939) reported five cases from Calcutta. (Total number of cases 9).

Punjab : Megaw and Gupta (*loc. cit.*) reported four doubtful cases from Lyallpur (reported by Civil Surgeons), Gupta (1935) reported a case in Lahore, and Bajaj (1939) reported six cases in Kangra. (Total number of cases 11).

United Provinces : Swarup (1930) described a case from Ballia.   (Total number of cases 1).

Hyderabad : Lowe (1931) reported 40 cases of pellagra in the patients of Dichpali Leper Hospital ; the first case observed was in 1925.   Thirty more cases were described from the same hospital by Lowe (1933).   (Total number of cases 70).

Bombay : Rajadhyaksha (1933, quoted by Stannus, 1936) described one case, Dhayagude and Khadilkar (1939) five cases from Bombay, and Mody (1935) one case from Poona.   (Total number of cases 7).

Madras : Raman (1933) described four cases in Guntur, the first case observed was in 1930.   Rau and Raman (1936) reported eight cases from Vizagapatam. The present series consists of 25 cases.   (Total number of cases 37).

II. CLASSIFICATION OF PELLAGRA.

The present method of classification of pellagra as primary and secondary is not satisfactory.   To avoid confusion, pellagra has been classified under three headings :—

Group 1.   *Primary pellagra.*—Cases with symmetrical exfoliative dermatitis and a distinct line of demarcation with or without the other symptoms of pellagra. Ten cases are included in this group.

Group 2.   *Primary pellagra* (with incidental disease).—The co-existent disease has practically nothing to do either with the onset or with the progress of the disease. Three cases are included in this group.   One was in a case of duodenal ulcer, the second in a case of cerebral thrombosis with hemiplegia, and the third in a case of stricture of the urethra.

Group 3.   *Secondary pellagra.*—Pellagra occurring in the course of other diseases.   Here the co-existent disease has either precipitated the onset of pellagra or made the condition of the patient worse.   Twelve cases are included in this group.   The associated conditions are :—

		Number of cases.
1.	Beri-beri	3
2.	Diabetes mellitus	1
3.	After gastrectomy for duodenal ulcer	1
4.	After gastro-enterostomy for duodenal ulcer	1
5.	Tubercular peritonitis	1
6.	Mental symptoms (? present before the onset of pellagra)	2
7.	Leprosy	2
8.	Carcinoma penis	1

III. REPORTS OF SIX CASES.

Group 1.—*Primary pellagra.*

A brief summary of the cases is given below :—

CASE No. 1.   Hindu male, aged 23 years, admitted on 19–1–37.   He gave a history of abdominal pain for four years ; indigestion, loss of appetite, and frequency of motions, for two years.   For the last three months his condition has become worse, and dyspnœa on exertion, œdema of the legs, appeared.   Physical examination : An emaciated individual, anæmic, tongue red, epithelium destroyed

(Plate XXXVI, fig. 1), salivation present, no jaundice. Liver and spleen normal. Circulatory and respiratory symptoms normal. Nervous system was normal except for the subjective sensation of tingling and numbness of the upper and lower extremities. Patches of exfoliative dermatitis were seen on the dorsum of both the hands, feet, on the back of the neck, and on the pinnæ of both the ears. Blood: R.B.C. 1·16 millions; W.B.C. 15,000 per c.mm.; hæmoglobin, 25 per cent. Colour index 1·1. Halometric reading 4·6. Blood smear showed anisocytosis, poikilocytosis, and macrocytosis; polymorphs 71 per cent; lymph. 18 per cent; mono. 8 per cent; eos. 3 per cent. Van den Bergh reaction: Direct positive delayed, faint; indirect positive faint. Fragility of the R.B.C. normal. Blood chemistry: Total proteins 5·4; albumin 2·2; globulin, 2·9, and fibrin 0·3 g.; cholesterol 53·49 mg.; Wassermann reaction: Negative. Blood-pressure 108/86. Urine: Normal. Motions: Loose, greenish yellow in colour, contained undigested material, no ova and no protozoa. Gastric analysis: Stomach empty in 45 minutes, total acidity very low, free HCl absent in all specimens, achylia. X-ray after barium meal: Stomach not empty in five hours; a long retro-cæcal appendix was seen in 24 hours' picture. Fat analysis of stools: Total fat 31·08; unsoaped fat 15·2 g.; free fatty acid 9·06 g.; neutral fat 6·14 g.; combined fatty acids 22·02 g. per cent. Electrocardiogram showed nothing abnormal. The patient was put on Marmite, milk, and bread diet with eggs, and liver soup by mouth and liver extract injections 2 c.c. every day, then on alternate days and afterwards twice a week. The condition gradually improved and the diarrhœa and dermatitis disappeared. After treatment: Blood count R.B.C. 2·88 millions. Hæmoglobin, 48 per cent. Colour index 0·8. W.B.C. 11,250; poly. 50 per cent; lymph. 12 per cent; mono. 12 per cent; eos. 26 per cent. Smear showed slight anisocytosis and poikilocytosis. Halometric reading 4·9. Blood chemistry: Total proteins 6·6; albumin 3·2 g.; globulin 3·1 g.; and fibrin 0·3 g., and cholesterol 134 mg. per 100 c.c. Gastric analysis: Stomach empty in one hour, free HCl absent in the resting juice, although total acidity was low, free HCl appeared in the later specimens showing a decided improvement over the previous condition. A second X-ray after a barium meal showed increased tonicity of the stomach wall. He put on 20 lb. and was discharged cured on 14–3–37.

CASE No. 2. C. S., Hindu male, aged 14 years, admitted on 16–7–38 with a history of pigmentation on the dorsum of both the legs, of six months' duration, more prominent for the last three months. Physical examination: A fairly well-nourished boy, not anæmic, sore-mouth and tongue (angular stomatitis). Spleen and liver normal. Respiratory and circulatory systems normal, blood-pressure 110·65. Nervous system exaggeration of deep reflexes, and a doubtful Babinski on the right side. Skin (Plate XXXVII, fig. 7): Exfoliative dermatitis with well-defined margins could be seen on the dorsum of the hands and feet. Blood count: R.B.C. 4·5 millions; W.B.C. 10,000; hæmoglobin 80 per cent. Smear showed anisocytosis, poikilocytosis, and a picture of microcytic anæmia. Halometric reading 5·2. Blood chemistry: Total proteins 4·9; albumin 2·9; globulin 1·7 and fibrin 0·3 g. per 100 c.c. Urine: Total ether and chloroform soluble porphyrins: 11·5 mg. per 100 c.c. Motions: No ova; no protozoa. Fat analysis of stools: Total fat 14·4 g., unsoaped fat 3 g.; free fatty acids 2·226 g.; neutral fat 0·774 g. and combined fatty acids 12·174 g. per cent. X-ray after barium meal: Nothing abnormal. He was put on liberal diet with eggs and liver soup and campolon injections 2 c.c. every alternate day. After treatment the dermatitis disappeared and the general condition improved. Electrocardiogram showed nothing abnormal. He was discharged cured on 6–8–39 before any further investigation could be done.

Group 2.—Primary pellagra (with incidental disease).

CASE No. 3. R. P., Hindu male, aged 50 years, coolie, admitted on 17–12–36 with indigestion and loss of appetite. He gave a history of duodenal ulcer for two years and pigmentation on the dorsum of both hands and feet for two months. Physical examination: A poorly-nourished anæmic individual; no jaundice or œdema; spleen and liver normal. Circulatory and respiratory systems normal. Nervous system: Slight anæsthesia of hands and feet. Skin: Exfoliative dermatitis on the dorsum of both the hands and feet. Blood count R.B.C. 2·2 millions; W.B.C. 5,000; poly. 70 per cent; lymph. 13 per cent; mono. 10 per cent; eos. 7 per cent; hæmoglobin 45 per cent. Smear showed anisocytosis, poikilocytosis, and a picture of microcytic anæmia. Blood chemistry: Total proteins 7·0; albumin 3·2; globulin 3·5 and fibrin 0·3 g. per 100 c.c. Gastric analysis (25–2–36) showed increase in both total and free HCl, suggesting cicatrizing duodeno-pyloric ulcer with obstruction. Barium meal confirmed the diagnosis. The patient was put on milk and bread diet with two eggs and 8 oz. of liver soup a day. Three weeks after admission the patient improved considerably and the dermatitis completely disappeared. After treatment (8–2–37): R.B.C. 3 millions; W.B.C. 7,000; hæmoglobin 80 per cent; poly. 80 per cent; mono. 7 per cent; lymph. 9 per cent; eos. 4 per cent; blood smear showed only slight anisocytosis, and poikilocytosis and a picture of normocytic anæmia. Halometric reading 4·9. Blood chemistry: Total proteins 5·7; albumin 4·0; globulin 1·3 and fibrin 0·4 g. per 100 c.c. Gastric analysis (27–1–37) showed a curve similar to the first. This patient was cured of pellagra and transferred to the surgical department. Operation confirmed the diagnosis of peptic ulcer.

Group 3.—Secondary pellagra.

CASE No. 4. P. J., Hindu male, aged 19 years, admitted on 23–8–38 for tingling and numbness of the lower extremities with slight œdema of the legs of one and a half months' duration. Physical examination : A moderately nourished individual with anæmia, angular stomatitis and superficial glossitis. Spleen and liver normal. Respiratory and circulatory systems normal. Nervous system : deep reflexes *lost*, superficial reflexes normal, and sensation to cotton-wool lost in both the upper and lower extremities. Skin showed exfoliative dermatitis with well-defined margins on the dorsum of both the hands and feet. Blood count : R.B.C. 4·8 millions ; W.B.C. 11,000 ; poly. 43 per cent ; lymph. 40 per cent ; mono. 2 per cent ; eos. 15 per cent ; hæmoglobin 80 per cent. Halometric reading 4·75. Smear showed anisocytosis, poikilocytosis, and a picture of normocytic anæmia. Blood chemistry : Total proteins 4·2 ; albumin 2·7 ; globulin 1·3 and fibrin 0·2 g. per 100 c.c. Van den Bergh reaction : Direct, positive delayed, indirect positive. Urine : Normal. Motions : no ova, no protozoa. Fat analysis of stools : Total fats 22 ; unsoaped fat 14·61 g. ; free fatty acid 5·9 g. ; neutral fat 8·71 and combined fatty acid 16·1 g. per cent. Gastric analysis showed nothing abnormal.

On the day of his admission his condition was typical of acute beri-beri. Slight œdema of the feet and a blood pressure of 110/10 with femoral arterial sounds were present. He was given 2 c.c. of Betaxin. Next day the œdema disappeared and the blood-pressure rose to 115/50 and in another twenty-four hours to 125/80. Electrocardiogram showed nothing abnormal. On 29–8–38 he was put on nicotinic acid (60 mg. a day) continuously for ten days. The improvement shown was at first only slight. At the end of the week the general condition improved and the dermatitis disappeared. On 11–10–38 he got slight œdema of the legs and was put on Betaxin injections every alternate day. A week later symptoms of pellagra re-appeared (exfoliative dermatitis). He was again put on nicotinic acid (90 mg. a day), for 3 days only since the stock was exhausted. He was getting in addition cod-liver oil and syrup ferri iodide. The dermatitis disappeared but symptoms of beri-beri persisted. He was discharged on 22–11–38.

CASE No. 5. S. S., Hindu male, aged 30 years, admitted on 5–4–38 for œdema of the feet, and dyspnœa on exertion of ten days' duration. This patient was originally seen on 18–9–37 in the out-patient department and was sent to the surgical department for operative treatment of duodenal ulcer. The diagnosis was confirmed both by fractional test meal and X-ray with barium. Operation on 13–10–37 revealed an ulcer in the first part of the duodenum and a partial gastrectomy was performed. He was discharged on 31–10–37 relieved of his symptoms. Physical examination : A poorly-nourished individual with anæmia and œdema of the feet. Spleen and liver normal. Circulatory system normal, except for a pulmonary systolic murmur. Respiratory and nervous systems normal. Exfoliative dermatitis was present on the dorsum of both the feet, and according to his statement similar patches were seen on the dorsum of both the hands and disappeared only a few days previously. Blood count : R.B.C. 3·7 millions ; W.B.C. poly. 50 per cent ; lymph. 15 per cent ; mono. 12 per cent ; eos. 23 per cent. Smear showed slight anisocytosis and a picture of macrocytic anæmia. Hæmoglobin 75 per cent, colour index 1. Van den Bergh reaction : Direct negative, indirect positive. Fragility of R.B.C. normal. Blood chemistry : Total proteins : 4·0 ; albumin 1·8 ; globulin 2·0 and fibrin 0·2 g. per 100 c.c. Gastric analysis :

	11-10-37		7-4-38		14-5-38	
	Total.	Free HCl.	Total.	Free HCl.	Total.	Free HCl.
Before meal ..	90	78	20	5	20	6
½ hour after ..	25	21	26	16	4	*Nil.*
½ ,, ,, ..	29	25	52	41	10	5
¾ ,, ,, ..	40	34	21	15	30	20
1 ,, ...	58	51	64	51	49	37
1¼ hours after ..	69	61	..	..	46	30
1½ ,, ,, ..	89	80	..	..	40	24
1¾ ,, ,, ..	97	87	..	..	..	..
2 ,, ,, ..	95	86	..	..	..	..
2¼ ,, ,, ..	66	55	..	..	..	..
2½ ,, ,, ..	53	43	..	..	..	..

The second and third gastric analyses were within normal limits. The patient was put on a liberal diet with two eggs and 8 oz. liver soup by mouth daily and Campolon injections 2 c.c. every day for the first one week, and later, on alternate days. As a result of treatment the general condition of the patient improved, and the dermatitis and œdema completely disappeared. He was discharged cured on 27–5–38.

CASE No. 6. A Hindu male, aged 35 years, admitted on 26–1–38. He had been in hospita for influenzal pneumonia in 1937 and then showed slight symptoms of mental deficiency. Physical examination : A poorly-nourished individual, anæmic, tongue moist and coated. Nervous system : Memory and intelligence poor ; speech sometimes vague and incoherent, deep reflexes exaggerated ; plantar response extensor on both sides ; no inco-ordination, but muscular power feeble ; slight temperature between 99°F. to 100°F. Spleen and liver normal. Circulatory and respiratory systems normal. Blood-pressure 100/78. Blood smear showed anisocytosis, poikilocytosis and a picture of microcytic anæmia. W.B.C. poly. 60 per cent ; lymph. 30 per cent ; mono. 3 per cent ; eos. 7 per cent. Urine : Normal. Motions : No ova, no protozoa. Blood chemistry : Total proteins, 3·0 ; albumin 1·3 ; globulin 1·4 and fibrin 0·3 g. per 100 c.c. Wassermann reaction : Negative both in blood and C.S.F. Skin : Exfoliative dermatitis with well-defined margins could be seen on the dorsum of both the hands and feet. He was put on a liberal diet with two eggs and 8 oz. liver soup daily by mouth. He was given Campolon 2 c.c. every day till 22–3–38, then every alternate day and then once a week and had his last injection on 11–4–38. On the whole he had 56 injections (112 c.c. of liver extract). His general condition improved and the dermatitis completely disappeared. The mental condition, however, remained the same. On 29–3–38 he went out of the hospital, roamed about the town and was brought back the same day. Later he could only be controlled with Hyoscine injections morning and evening. He was transferred to the mental hospital on 19–5–38 and died there on 10–9–38.

IV. SYMPTOMATOLOGY.

All types of cases from the mildest case with only a small patch of dermatitis to the most severe type with extreme emaciation, œdema, anæmia, diarrhœa, and exfoliative dermatitis are represented in this series. All cases of primary pellagra (with incidental disease), a few cases of primary and secondary pellagra came to the hospital for some other complaint ; and the diagnosis of pellagra was made either accidentally or in the routine examination of the patient.

1. *Skin.*—Exfoliative dermatitis with well-defined margins could be seen in all the cases. Two cases showed only small patches 25 mm. in diameter and one case showed extensive exfoliation, extending over the extensor aspect of both the upper and lower extremities, and the whole of the back with definite line of demarcation on either side of the chest. The patches have a definite line of demarcation and are always symmetrical, but in some cases the size might be bigger on one side than the other ; the symmetry may be manifested only after some time. The patch always starts as a red hyperæmic area ; after a few days it becomes dark red in colour, then light brown, then dark brown and finally black in colour. The whole process usually takes two to three weeks. After this, exfoliation takes place in large plaques and when the dark scales are removed, the area left behind is paler than normal. Extension of the patch occurs at the periphery, and when a definite line of demarcation is formed no further extension of the patch occurs.

The commonest sites of the lesions are on the dorsum of hands and feet. Usually the patch is limited to the wrist above and proximal phalanges below. Sometimes it might extend to the level of the elbow. In extremely rare cases, the lower limit comes down to the base of second phalanges. In the lower extremities the upper limit usually stops short at the level of the ankle but in rare cases it may extend to the level of the knees. Patches are also seen on the neck, face, thighs, and back.

Histopathology of the skin.—Sections of skin were examined from five cases.

Epidermis.—The horny layer invariably shows some degree of hyperkeratosis without parakeratosis. The stratum granulosum is thin and stretched out. The stratum spongiosum in the early stages shows slight proliferative activity with spongiosis and a moderate amount of acanthosis. In the chronic cases this layer becomes thinner and the papillæ tend to disappear. The stratum germinativum shows general increase of pigment which extends to two or three layers. The pallor seen by the naked eye after desquamation seems to be due rather to the thinness of the cuticle than to the diminution of pigment.

Dermis.—The superficial layer shows increased activity of chromatophores. In later stages as mentioned above the papillæ tend to disappear. In some cases there is œdema of the loose connective tissues of the deeper layers of the dermis, while others show hyalinization of the collagen bundles. Infiltration especially with lymphocytes and mononuclear cells are found in small circumscribed areas in relation with smaller capillaries and lymphatics. The lesions are slight and have to be looked for. This cannot be regarded as a diffuse inflammatory granulomatous condition of the skin.

In some cases hair appears thinned out and the follicles atrophied. As a rule sebaceous and sweat glands are normal, but in advanced cases they show slight atrophy.

2. *Alimentary system.*—Indigestion and loss of appetite was complained of by 17, diarrhœa by three, and constipation by three patients. Angular stomatitis and superficial glossitis were present in a mild degree in 15 cases and were severe in three cases.

3. *Nervous system.*—Subjective sensation of tingling and numbness of the extremities, anæsthesia over the dorsum of both feet, and exaggeration of deep reflexes, with or without Babinski's sign, were the symptoms and signs manifested. Three cases showed the sign and symptoms of beri-beri, two cases with leprosy showed anæsthesia of leprosy, and one case extensive involvement of the ulnar and external popliteal nerve of the left side. Two cases showed mental symptoms and in one of them these were so severe that he had to be transferred to the mental hospital. The question arises whether the mental symptoms can be attributed to pellagra. In one case the mental symptoms may have been of long duration and the onset of pellagra may have precipitated the acute manifestations.

4. *Circulatory system.*—Two cases of secondary pellagra with beri-beri had cardiac enlargement with mitral systolic murmurs and low systolic and diastolic pressure with increased pulse pressure. In the one case of primary pellagra (with incidental disease) the heart was hypertrophied with a systolic murmur in the mitral area and high blood-pressure (182/112). Otherwise for occasional hæmic murmurs heard in the pulmonary area nothing abnormal could be made out. Electrocardiograms were taken in six cases, three of primary pellagra, one of primary pellagra (with incidental disease), and two of secondary pellagra. The case of secondary pellagra with diabetes mellitus showed regular sinus rhythm with prominent P wave in lead two and prolongation of P-R interval, evidence of myocarditis. One

case showed sinus tachycardia at first but later a normal electrocardiogram was obtained.

5. *Respiratory system.*—One case showed signs of tubercular infiltration of the left apex.

6. *Œdema*—Was present in six cases; of these three were associated with beri-beri and in the other three the causative factor was in all probability diminished plasma proteins.

V. Blood picture.

	Primary pellagra.	Primary pellagra (with incidental disease).	Secondary pellagra.	Totals.
Macrocytic anæmia ..	2	0	3	5
Normocytic anæmia ..	0	0	1	1
Microcytic anæmia ..	4	2	2	8
Normal	1	1	0	2
Blood smear not examined.	3	0	6	9
Totals ..	10	3	12	25

The blood was examined in 16 out of the 25 cases. Two cases showed normal blood picture, five cases showed macrocytic, one normocytic, and eight microcytic, anæmia. The diagnosis of the nature of the anæmia was based on colour index, halometer reading, and microscopic examination, of the smear. The fragility of the cells was normal in all the cases examined. The only constant finding was a slight leucocytosis. In some cases there was a relative decrease of polymorphs and a definite increase of eosinophils, which came down as a result of treatment. When large doses of liver extract were given there was definite increase of eosinophil cells with a relative fall of polymorphs.

VI. Biochemical investigations.

(1) *Blood proteins* were investigated in 18 cases and in six cases estimations were done before and after treatment. Total proteins were low except in two cases. In both these the albumin content was low and the deficiency was made up by increase in globulin. Albumin was low in all the cases and in two below 2 g. per 100 c.c. Globulin was usually low but was above 3 g. per 100 c.c. in two cases. Fibrin was normal. After treatment with a liberal diet with eggs and liver soup the blood proteins rose to normal, and the increase was mainly in the albumin content. In the cases where the globulin was abnormally high, the total proteins diminished

as a result of treatment and resulted in the normal albumin/globulin ratio. In two cases of secondary pellagra, one with beri-beri and the other with nerve leprosy, the proteins remained low after treatment.

(2) The *blood sugar* was estimated in 12 cases and was within normal limits, except in two cases where diabetes mellitus and renal glycosuria were present.

(3) The *blood urea* was normal in 17 cases investigated.

(4) The *blood calcium and phosphorus* were estimated in 15 cases and was normal except in one case in which both were low (7 mg. and 2·14 mg., respectively).

(5) *Bilirubin.* The van den Bergh reaction was done in 10 cases. In four cases, direct delayed, and indirect positive ; in four cases direct negative indirect positive and in the other two both direct and indirect negative. In all the cases the amount was too small for quantitative estimation.

(6) The diastase in *urine* was normal in four cases.

(7) Porphyrins in urine : In one case ether soluble porphyrins only were estimated (4·5 mg. per 100 c.c.) and in the other case both ether and alcohol soluble porphyrins were estimated (11·5 mg. per 100 c.c.).

(8) *Fat analysis of stools.*—Estimation of fat in the stools was done in five cases, three primary and two secondary pellagra. One case showed increase in both the split and unsplit fat ; one case showed increase in the total fat with relative increase of neutral fat, a second analysis showing a normal fat content ; one case showed normal fat content with a relative increase of neutral fat and one case showed increase in total fats with defective absorption.

(9) *Gastric analysis.*—Fractional test meals were done in 14 cases. Acidity was low in five, normal in three, and high in six cases. Advanced cases showed low acidity with absence of free HCl.

VII. X-ray after barium meal.

Ten cases were investigated radiologically after a barium meal. One case showed increased tonicity of the stomach wall after treatment. No characteristic abnormalities were found.

VIII. Diagnosis.

The classical signs of well-developed pellagra are : (1) skin lesions as manifested by symmetrical exfoliative dermatitis with well-defined margins ; (2) gastro-intestinal symptoms such as angular stomatitis, superficial glossitis, and diarrhœa ; (3) nervous signs such as peripheral neuritis and degenerations of the spinal cord which are possibly due to an associated vitamin-B_1 deficiency ; and mental symptoms. Diarrhœa and mental symptoms are usually seen only in the later stages of the disease. The earliest manifestation is the dermatitis, and this is the main diagnostic feature of pellagra. The presence of diarrhœa and nervous symptoms are additional factors and should not be considered essential diagnostic points. Symmetrical exfoliative dermatitis with well-defined margins occurring on the dorsum of both the hands and feet is diagnostic of pellagra.

The conditions usually mistaken for pellagra are :—

(1) *Oro-genital syndrome* (Nair, 1939).—Angular stomatitis and superficial glossitis of pellagra simulates oro-genital syndrome but the presence of symmetrical exfoliative dermatitis distinguishes the latter condition. Pellagra and oro-genital syndrome may occur together.

(2) *Ichthyosis.*—The exfoliation of the skin is present all over the body and there is no definite line of demarcation characteristic of pellagra.

(3) *Exfoliation of the skin in the cold weather* (November, December, January and February).—The scales are very small, the colour is not so dark as in pellagra, the line of demarcation is not definite and exfoliation is more marked on the palms.

(4) *Sprue.*—The diarrhœa and anæmia of pellagra can be mistaken for sprue. In such conditions exfoliative dermatitis is the only distinguishing feature, and when the dermatitis of pellagra has disappeared it is impossible to distinguish one condition from the other.

(5) *The mental condition* may be suggestive of general paralysis or other nervous diseases, but the skin lesions and digestive disturbances should make the diagnosis clear.

IX. TREATMENT.

(1) *Diet.*—Except in cases with diarrhœa, the patients were put on ordinary Indian diet, or milk and bread diet.

(*a*) Composition of ordinary Indian diet.

Rice	1 lb.
Bread	4 oz.
{Butter	1 oz. }
{or Ghee	$\frac{1}{2}$ oz. }
Coffee (milk 3 oz. ; sugar 1 oz.) ..	2 pints.
Butter milk	$\frac{1}{2}$ pint.
Gingelly oil	$\frac{3}{4}$ oz.
Plantain	1
Sugar	1 oz.
Mutton	4 oz.
Potatoes or vegetables	4 oz.

Mutton 4 oz. may be substituted by dhal 4 oz., or fish 4 oz., or eggs 2.

(*b*) Composition of milk and bread diet.

Milk	$1\frac{1}{2}$ pints.
Bread	12 oz.
Butter	1 oz.
Sugar	2 oz.
Coffee	1 pint.
Plantain	2

In addition to these diets the patients were given 2 eggs and 8 oz. of liver soup daily. In one case the patient took his usual diet at home.

(2) *Liver.*—Liver was administered either by mouth or by injections. Eight oz. of liver either in the form of curry or soup was given to every patient except

in three cases. In serious cases Campolon 2 c.c. (intramuscularly) was given daily for the first few days, then on alternate days, then twice a week and finally once a week. The maximum amount of liver extract given to a case was 112 c.c.

(3) *Nicotinic acid.*—(3 Pyridine-Carboxylic-acid).

Seven cases, four of primary and three of secondary pellagra, were treated with doses of 60 mg. to 90 mg. a day. Three patients showed considerable improvement, and in one the improvement was remarkable and there was complete disappearance of the dermatitis in seven days. In two cases the improvement was in no way better than that shown by the other methods of treatment. One case, complicated by beri-beri, did not respond.

(4) *Iron.*—Ferri et ammonium citras (60 to 90 grains) was given in cases of microcytic and normocytic anæmias until definite improvement was shown.

(5) *Hydrochloric acid.*—Acid hydrochloric dil. (20 min. t. d. s.) was given in all cases showing absence or deficiency of HCl. One case did not show any increase in HCl, even after the administration of acid for a long time.

X. Discussion.

It is now more than two hundred years since pellagra was first described as a clinical entity and still many medical men consider the disease to be non-existent in India. Pellagra is one of the diseases in which the diagnosis is very easy if one knows what it is, and if one is on the look out for it. After several years of experience Stannus has studied pellagra from an international point of view and has published his monumental work (1936-37) with 273 references. Since the publication of this series of articles, a definite advance has been made in the treatment of pellagra by the introduction of nicotinic acid.

Geographical distribution of pellagra.—Pellagra has a very wide distribution and if carefully looked for, can be seen in each and every country. As far as my observation goes, pellagra in India is seen only in the two areas where beri-beri is endemic. But one is not justified in drawing this conclusion from a study of the two endemic areas. In all probability further investigation might show that the disease is more widely distributed in India, especially in the Madras Presidency.

Epidemiology.

(1) *Incidence.*—The majority of patients admitted to the Hospital come from the Koraput and Ganjam districts of Orissa Province and from Vizagapatam, East and West Godavary and Kistna districts of the Madras Presidency.

All the cases except one were from the Vizagapatam district.

	1937.	1938.	TOTALS.
Total admissions	7,416	8,165	15,581
Admissions in medical wards ..	1,402	1,611	3,013
Number of cases of pellagra ..	10	11	21

The incidence of pellagra was 0·65 per cent of admissions to the medical wards.

(2) *Age.*—Pellagra has been described in young and old. In this series the youngest patient was a girl of 13 years and the oldest a man of 70 years.

(3) *Sex.*—In the present series only two cases occurred in females. I have come across two more cases in females. Thus there is a decided preponderance of males over females. Males expose themselves more to sunlight than women. Whether this has anything to do with the dermatitis of pellagra is difficult to say.

(4) *Seasonal variation.*—Almost all the cases in this series were admitted in the months of November, December, January, and February. Only in the year 1938 were a few cases seen in the months of July and August. It has been suggested that during the cold weather people sit in the sun and this exposure to the sun's rays is responsible for greater incidence of pellagra in this season of the year. People cover themselves up when sitting in the sun, and the only part that is exposed is a small portion of the hands and feet. The upper limit of the line of demarcation of the pellagrous dermatitis was higher than the areas exposed to the sun.

Maize theory.—This theory is the oldest and has many supporters. In Italy and France the introduction of maize has been followed by the appearance of pellagra. In all the cases that I have come across maize can be excluded as the causative factor in the production of pellagra.

Protein deficiency.—All the cases in this series except two were very poor and could not afford even a single meal a day. The diet of the patients probably consists of :—

Rice (milled)	16 oz. to 20 oz.
Ragi	4 oz. every day or every alternate day.
Dhal	2 oz. a week.
Vegetables: brinjals, cucumber, green beans.	2 oz. once a week.
Butter milk	6 oz. occasionally.
Fish or mutton	4 oz. once a week.

Estimation of blood proteins showed a constant diminution except in two cases. The reduction in the albumin was the most prominent feature; and in two cases it went down to the abnormally low figure of less than 2 mg. per 100 c.c. From these observations one is convinced that diminution of plasma proteins is a definite contributory factor in the production of pellagra.

Defect in digestion of proteins is practically out of question. Normal urinary diastase in two of the fairly-advanced cases eliminates a pancreatic defect. Defect in absorption is certainly present and is marked in cases with diarrhœa.

Vitamin deficiency.—In 1912 Deeks and Funk (Manson-Bahr, 1935) suggested that pellagra is due to vitamin deficiency. This vitamin is contained in the vitamin-B complex. It is otherwise called vitamin B_2 or vitamin G, or ' pellagra-preventing factor '. Goldberger and Wheeler in America confirmed the hypothesis of vitamin deficiency. Goldberger and Tanner finding this vitamin efficient as a

prophylactic and curative agent against black tongue in dogs used it in human pellagra and found it effective.

A study of both beri-beri and pellagra in two areas (Guntur and Vizagapatam) was made. During the course of five years in Guntur there were more than 500 cases (actual figures not available) of beri-beri; but only four cases of pellagra. In Vizagapatam the number of cases of beri-beri are few and pellagra more than in Guntur. Are the relative proportions of the anti-neuritic and pellagra-preventing factors of the vitamin-B complex different in the diets of the two areas? Both beri-beri and pellagra occurred together in three cases, and in all three beri-beri was the primary condition and pellagra was secondary. In one case the symptoms of pellagra re-appeared even after treatment with vitamin B_1 and nicotinic acid. The only conclusion that one can draw from these findings is that deficiency of vitamin B_2 alone is not the sole cause of pellagra.

Deficiency of anti-anæmic principles.—Castle formulated the theory that pellagra, sprue, and pernicious anæmia, were due to deficiency of anti-anæmic factor. In cases of pellagra with macrocytic anæmia all the three factors, viz., deficiency of intrinsic factor, deficiency of extrinsic factor, and defect in absorption, are present either singly or in combination. In cases with normocytic and microcytic anæmia the defect is in the extrinsic factor and absorption. Therapeutic tests with injections of liver extract will convince one that there is a defect of anti-anæmic principle in pellagra.

Dermatitis of pellagra.—The first question that has to be decided is whether the condition is an inflammatory or a degenerative lesion. From a study of the microscopic appearances of the skin there is slight inflammatory infiltration of the dermis with lymphocytes and mononuclear cells. Degenerative changes are seen in the later stages. Does the distribution of dermatitis suggest a trophic disturbance? In the only case of hemiplegia in this series the patches were symmetrical.

Gastric lesions in relation to pellagra.—Deficiency of acidity with absence of free HCl in pellagra is a well-established fact (Niles, 1923). Whether this condition is a contributory factor or not in the production of pellagra is a disputed point. Two cases showed definitely that a deficiency of HCl as a result of gastrectomy and gastro-enterostomy may be responsible for the production of pellagra. Petri, Norgaard and Bing (1938) demonstrated clinical and pathological changes similar to those of pellagra produced by gastrectomy in young pigs. In advanced stages of pellagra there is always a deficiency of total acidity with absence of free HCl. Pellagra is also seen in cases of active duodenal ulcer.

Nicotinic acid and pellagra.—In 1911 Funk (*Brit. Med. Jour.*, 1939) isolated a substance that cured polyneuritis in experimental animals which was found to be a compound of nicotinic acid with a pyridine base. Since nicotinic acid was not the anti-neuritic factor he did not proceed further. In 1937 Elvehjem and his associates (*Brit. Med. Jour.*, *loc. cit.*) found that liver extract cured black tongues in dogs and nicotinic acid is the active principle or the 'pellagra-preventing factor' in the liver extract. Later nicotinic acid has been used in the treatment of pellagra in man and has been found to be very efficient. Recent American literature is full of articles advocating the use of nicotinic acid in the treatment of pellagra (Shattuck, 1938; Spies, 1938; Spies *et al.*, 1938), and dramatic results are

PLATE XXXVI.

Fig. 1. (Case 1.) Angular stomatitis and superficial glossitis.
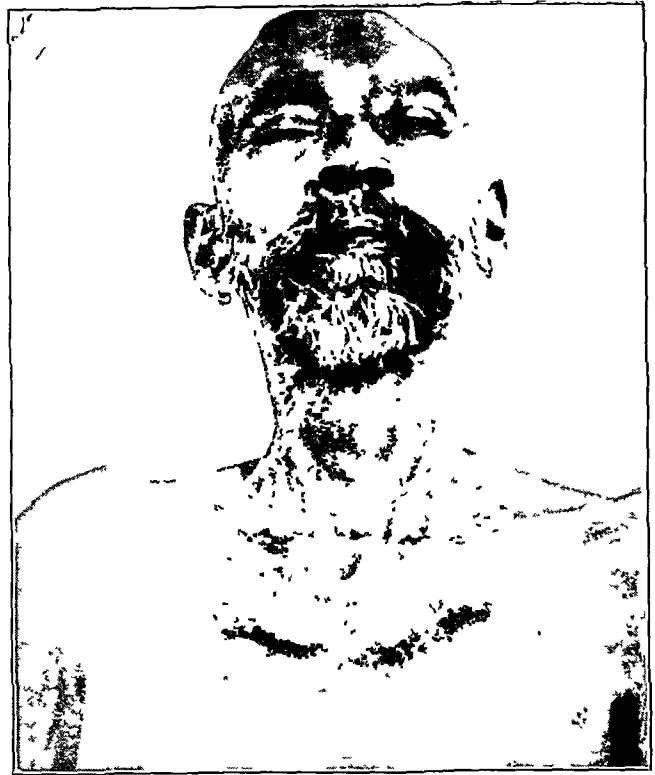
Fig. 2. Dermatitis around the neck.
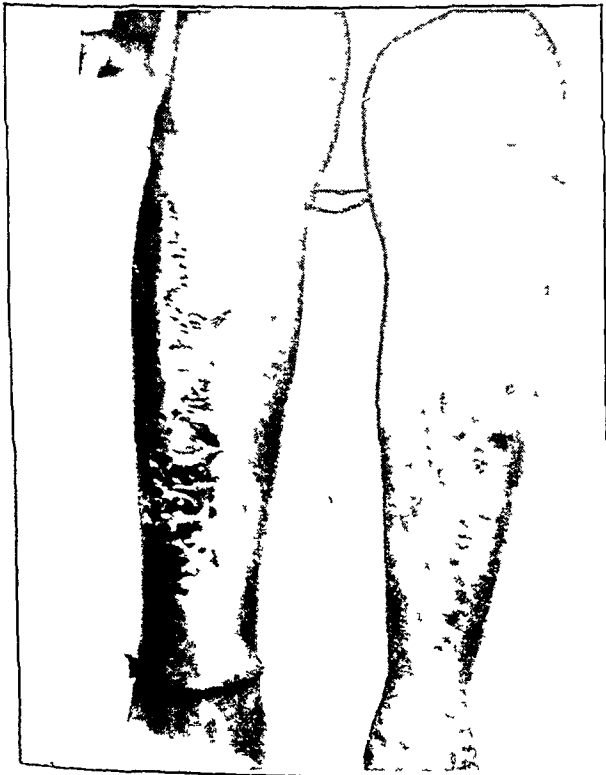
Fig. 3. Patches on the dorsum of both the legs.

Fig. 4. Dermatitis on the dorsum of both the feet.

PLATE XLXVII.

Fig. 5. Dermatitis on the dorsum of both the hands.

Fig. 6. Dermatitis on the inner aspect of the right thigh.

Fig. 7. (Case 2.) Dermatitis on the dorsum of both the feet; later view.

Fig. 8. Dermatitis of the back. Side view showing line of demarcation.

PLATE XXXVIII.

Fig. 9. Microphotograph (low power) showing hyperkeratosis, inflammatory infiltration of the dermis and intact sebaceous and sweat glands.
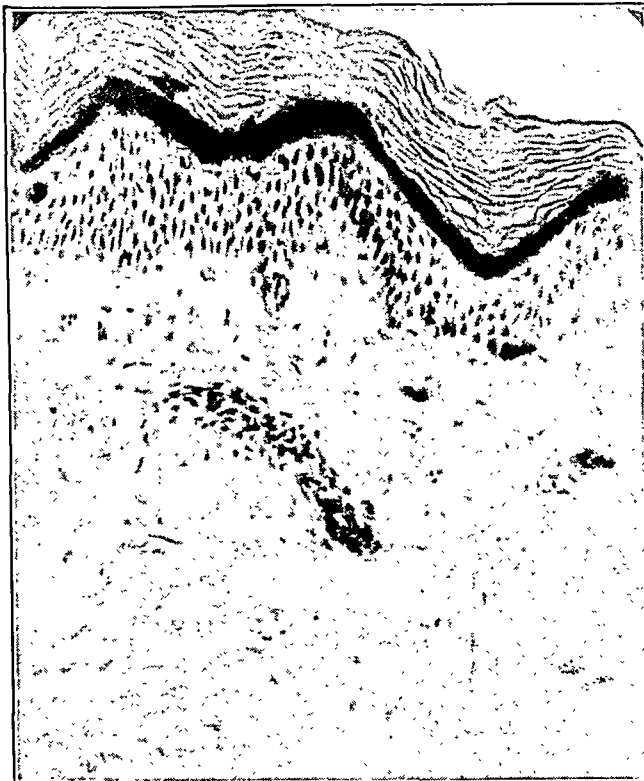
Fig. 10. Microphotograph (high power) showing hyperkeratosis spongiosis and inflammatory infiltration of the dermis.

Fig. 11. Microphotograph (low power) showing hyperkeratosis; slight flattening of the papillæ; inflammatory infiltration and œdema of the dermis. There is definite increase of pigment in chromatophores.
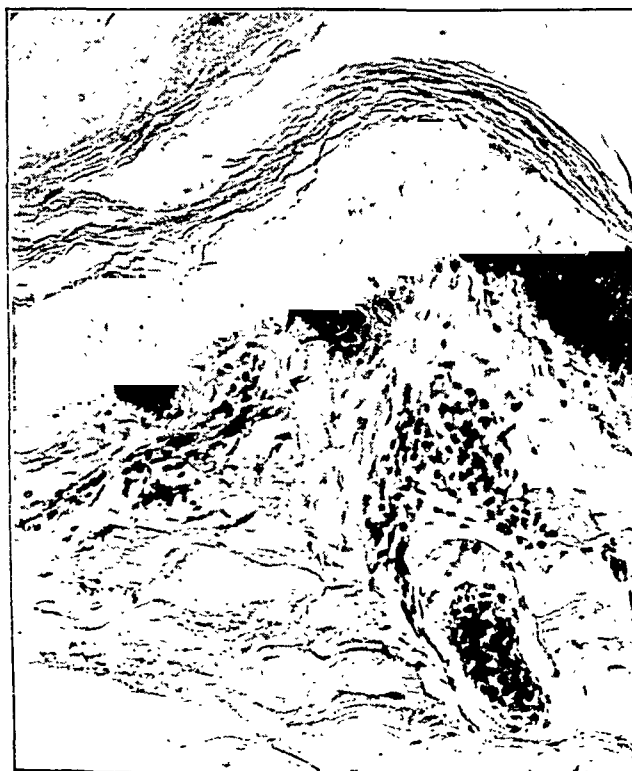
Fig. 12. Same as Fig. 11 (high power)

reported within 24 hours after its administration. Swaminathan (1938) working on the nicotinic-acid content of foodstuffs found the lowest value for maize and highest for yeast. My experience is restricted to the treatment of seven cases. The dosage adopted was from 60 mg. to 90 mg. a day by mouth. Larger doses were not given for fear of toxic symptoms. Three patients showed improvement and in one of them the improvement was remarkable. In two cases, the improvement was not in any way better than that obtained by the other methods of treatment. In one case nicotinic acid has been a failure. The maximum dosage was 1,080 mg. in twelve days. Assuming that nicotinic acid is the pellagra-preventing factor in liver extract, one has to take into consideration the other fractions also in the liver extract. Crude liver extract contains (1) an alcohol-soluble fraction necessary for the macrocytic anæmia of pellagra, (2) a water-soluble fraction necessary for the cure of microcytic anæmia, (3) an anti-toxic principle necessary to neutralize the toxins if any, and (4) an unknown fraction. The more crude the liver extract the more efficient it is in the treatment of pellagra. In one case administration of nicotinic acid although producing temporary improvement in the symptoms did not prevent their recurrence. Two cases (not included in this series) were given 50 mg. intramuscularly and the results were better than by oral administration. There were no toxic symptoms in any of the cases.

XI. Conclusions.

1. Pellagra has a wide range of distribution. Vizagapatam where rice is the main article of diet is an endemic focus for pellagra.

2. Pellagra has been classified under three headings : (a) primary pellagra, (b) primary pellagra with incidental disease, and (c) secondary pellagra.

3. Symmetrical exfoliative dermatitis with well-defined margins was the main diagnostic feature of pellagra.

4. All the three types of anæmia—macrocytic, normocytic, and microcytic—were found to occur in the cases observed. The majority showed microcytic anæmia. Normal blood picture was restored after treatment.

5. Total blood proteins were low, especially the albumin. The cholesterol was decreased. As a result of treatment both proteins and cholesterol were restored to normal.

6. Low acidity with absence of free HCl was a feature in some cases. Gastrectomy in one case and gastro-enterostomy in another was responsible for pellagra. Increased acidity also can occur in pellagra.

7. Increase in total fat with a relative increase of both neutral and split fat was a constant finding.

8. Treatment consisted in a liberal protein diet, with eggs and liver soup by mouth ; iron in cases showing microcytic and normocytic anæmia ; hydrochloric acid in cases showing low acidity with absence of free HCl and intramuscular injections of liver extract 2 c.c. every day or on alternate days depending upon the condition of the patient.

9. Nicotinic acid by mouth was tried in seven cases, with definite improvement in three cases.

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URINARY CLEARANCE OF FREE CHLORAL IN NORMAL AND LIVER-DAMAGED DOGS AND THE POSSIBILITY OF USING IT AS A TEST FOR LIVER FUNCTION.

BY

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THE functions of the liver are many and varied and hence the measurement of the functional efficiency of the organ has always been a most difficult task for laboratory workers. Several of the specific functions of the organ have been utilized from time to time as a means for detecting the presence of hepatic damage and, in some instances, of gauging the extent to which such damage has occurred. Tests based on the glycogenic function, e.g., the lævulose or galactose tolerance tests, on the power of withdrawal of toxin, e.g., dye tests (phenoltetrachlorphthalein or bromsulphalein test), on the formation of bile pigments, e.g., van den Bergh test, etc., are fairly well known and have been commonly used in hospitals and clinics. A number of tests based on the de-toxicating function of the liver have also been devised, e.g., phenol-conjugation test (Foster and Kahn, 1916), camphor test (van-Dooren and Destrèe, 1924), aspirin test (Beaumont and Dodds, 1931), etc., but these are rarely used in clinical practice. None of the numerous functional tests so far described, however, have been shown to be absolutely reliable and satisfactory, and the search for a suitable test still continues. Of late, the researches of Quick (1933) and Boyce and McFetridge (1938) have drawn attention to the possibility of using the hippuric-acid conjugation mechanism of the liver as a measure of the efficiency of the organ. While engaged in studying certain phases of the toxicity of chloral hydrate in dogs, we (Mukerji and Ghose, 1939) have made some observations regarding its urinary elimination in normal and liver-damaged animals which promise to offer a new and fairly sensitive test for gauging the de-toxicating efficiency of the liver through its glucuronic-acid conjugation mechanism.

One of the primary mechanisms through which the liver de-toxifies poisons, both of metabolic and extraneous origin, is its power of 'conjugation' whereby toxic products are rendered non-toxic by combination with a variety of substances,

of which glucuronic acid is the most important. This 'conjugation function' of the liver is brought into play when certain drugs, e.g., chloral hydrate, aspirin, menthol, etc., are introduced into the body. Pharmacologists have shown that chloral-hydrate administration in ordinary doses results in its primary conversion into trichlorethyl alcohol, which is later conjugated with glucuronic acid and is excreted as the non-toxic urochloralic acid according to the following reaction :—

$$CCl_3.CHO \longrightarrow CCl_3.CH_2OH + CHO.(CHOH)_4.COOH \longrightarrow CCl_3.CO(CHOH)_4.COOH.$$
(Chloral) (Trichlorethyl (Glucuronic acid) (Urochloralic acid)
alcohol)
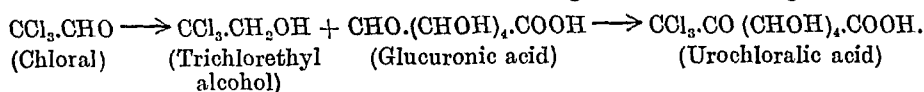
This method of excretion as urochloralic acid accounts for nearly the whole of the chloral administered, though a small part of it may be excreted unchanged or as inorganic chlorides. Because conjugation plays such a prominent part in determining the ultimate fate of chloral hydrate the integrity and efficiency of the liver, the only site of glucuronic-acid formation and conjugation (Hemingway *et al.*, 1934) assumes supreme importance. If this organ fails to function properly and to elaborate glucuronic acid to the same extent as under normal conditions, it is conceivable that chloral-hydrate conjugation may not take place with as much facility and with the same degree of completeness as before, and free chloral, which has escaped conjugation, may make its appearance in the urine. An estimation of this therefore is likely to afford an idea of the functional state of the organ. The following experiments have been planned to prove the above hypothesis.

METHODS.

Animals and their treatments.—Young healthy dogs weighing on an average between 4·0 kilo and 6·5 kilo were selected and were placed under identical conditions in metabolism cages provided with platforms especially designed for urine collection. Cooked meat, boiled rice, and bread crumbs formed the chief items in the daily diet which was kept as constant as possible during the whole period of experimentation. Water was allowed freely 4 to 5 times a day but no source of water allowed to be retained inside the cages to avoid an increase in the total urine volume due to accidental spilling by the animals. Female dogs were especially chosen to prevent urination outside the cages. A week was allowed for the animals to get used to the laboratory conditions before experiments were started.

Altogether 8 dogs were used. These were divided into three batches. Four dogs (Nos. 3, 5, 7, and 9) served as controls and in these 200 mg./kg. of chloral hydrate was administered by stomach-tube and the 24-hour urine was analysed for the presence of free chloral the next day. In other 4 dogs, liver damage was brought about by the oral administration of carbon tetrachloride. In dogs (Nos. 4 and 6) after the normal response to administered chloral hydrate (200 mg./kg.) was determined by estimating free chloral in urine, only a very mild grade of damage was induced by the administration of one or two doses (1 c.c. to 2 c.c. per kilo) of carbon tetrachloride. In the remaining two animals (Nos. 1 and 2) a very chronic type of damage was caused by the intermittent administration of the drug (0·25 c.c to 2 c.c. per kilo) every second or third day for 4 to 5 months according to the method of Lamson and Wing (1926). In all these 4 liver-damaged animals, chloral hydrate was again administered in identical dosages as in the control animals and their urine samples tested for the presence of free chloral.

Analytical methods.—The method described by Friedman and Calderone (1934) was used. The colour reaction which forms the basis of the test is given only by compounds of the R-C-halogen₃ group. It is therefore applicable only to free chloral or at most to chloral which has been very little modified. The presence of CCl_4, even if it is excreted in the urine in minute traces which is not usual (Robbins, 1929), is not likely to affect the result. When conjugated with glucuronic acid, chloral does not develop the pyridine colour. The test, in brief, is as follows :—

In a test-tube are placed 2 c.c of NaOH (4 per cent) solution, to which are added 1 c.c. pyridine (colourless) and then 4 c.c. urine. In a similar tube are placed the NaOH and pyridine and 4 c.c. of a solution of chloral hydrate containing 0·05 mg. to 0·025 mg. per c.c. (1 : 20,000 to 1 : 40,000) as standard. The two tubes are dipped in a water-bath for 1 minute and then cooled in an ice-bath for 1 minute. Six c.c. of water are then added to each and the solutions compared for intensity of colour in a colorimeter within 10 minutes. The pink colour which develops is often masked by the yellow pigment of the urine, rendering comparison with the standard difficult. A modification which was found convenient was the shaking of the urine with a small quantity of animal charcoal for about 15 minutes, allowing it to stand for another 15 minutes and then filtering. More or less complete de-colorization occurs and the urine can then be used for carrying out the test as described.

<div align="center">EXPERIMENTAL RESULTS.</div>

The average excretion of free chloral following the administration of 200 mg./kg. of chloral hydrate in apparently normal dogs is shown in the Table I. It will

<div align="center">TABLE I.</div>

Showing excretion of free chloral hydrate in 24-hour urine following oral administrations of the drug (dose 200 mg./kg.) in apparently normal dogs.

Dog number.	Weight in kg.	TOTAL EXCRETION OF FREE CHLORAL HYDRATE IN 24-HOUR URINE, FOLLOWING THE ADMINISTRATION OF 200 MG./KG. OF THE DRUG (FIGURES GIVEN IN MG.).						
		Experiments.						
		1	2	3	4	5	6	7
3	4·0	1·5	*Nil*	1·5	4·5	*Nil*	*Nil*	3
*4	5·5	*Nil*	1·25	*Nil*	..	..	..	..
5	4·7	5	2	*Nil*	*Nil*	2	3·5	*Nil*
*6	6·4	*Nil*	3·7	*Nil*	..	..	..	..
7	5·9	4·9	5·4	4·8	..	..	..	..
9	6·3	4·5	5·7	3·9	..	..	..	..

<div align="center">* Also included in Table II.</div>

be seen that the figures range between *nil* and about 6 mg. in 24-hour specimens (*see* bottom of Graph 2). Either there is no development of pink colour in the test-tube or a very faint pink colour is all that is noticeable in the majority of experiments.

Table II shows the average excretion in four dogs which received hepatoxic doses of carbon tetrachloride. The column on the top represents the excretion in 2 dogs where only one or two doses of carbon tetrachloride (1 to 2 c.c. per kg.) were administered. According to Gardner *et al.* (1925) fatty infiltration and early central necroses resembling the liver changes in acute yellow atrophy could be produced within 24 to 48 hours by this drug in doses of 0·25 c.c. to 4·0 c.c. per kilo. In dog No. 4, which previous to the initiation of liver damage showed only negligible excretion of chloral hydrate (*see* Table I), a significant increase of free chloral in urine after carbon-tetrachloride damage was easily evident. This increase was maintained for several days. On the 12th day, the dose of carbon tetrachloride was repeated and when the liver was exposed to this fresh dose of the poison, there was further increase of the free chloral in the urine. On allowing sufficient rest to the organ, there is a tendency for the damaged liver to undergo repair and regeneration and

TABLE II.

Showing excretion of free chloral hydrate in 24-hour urine after oral administration of the drug (200 mg./kg.) in dogs with acute and chronic liver damage.

Dog number.		Weight in kg.	TOTAL EXCRETION OF FREE CHLORAL HYDRATE IN 24-HOUR URINE AFTER 200 MG./KG. OF THE DRUG (FIGURES IN MG.).											
			Experiments.												
			1	2	3	4	5	6	7	8	9	10	11	12
Acute liver damage. {	4	5·5	8·5	15	10	15	14	15	8	6	..	..	..	..
	6	6·4	13·5	14	12	..	..	..	..	..	..	..	..	..
Chronic liver damage. {	1	6·3	30	25	17	14	20	14	16	19	21	18	20	20
	2	6·1	25	17	20	12	12	18	12	12	14	14	15	18

In dogs Nos. 1 and 2, CCl$_4$ was administered every alternate day throughout the period of experiments. In No. 4, CCl$_4$ was only administered before the 1st and the 4th experiments. In No. 6, CCl$_4$ was only administered once before the first experiment.

this is reflected in the gradual decrease of the free chloral elimination (*see* Graph 1). Dog No. 6 also showed an increase after the first dose of CCl_4 but this has, as yet, not been followed up to the same period as dog No. 4.

GRAPH 1.
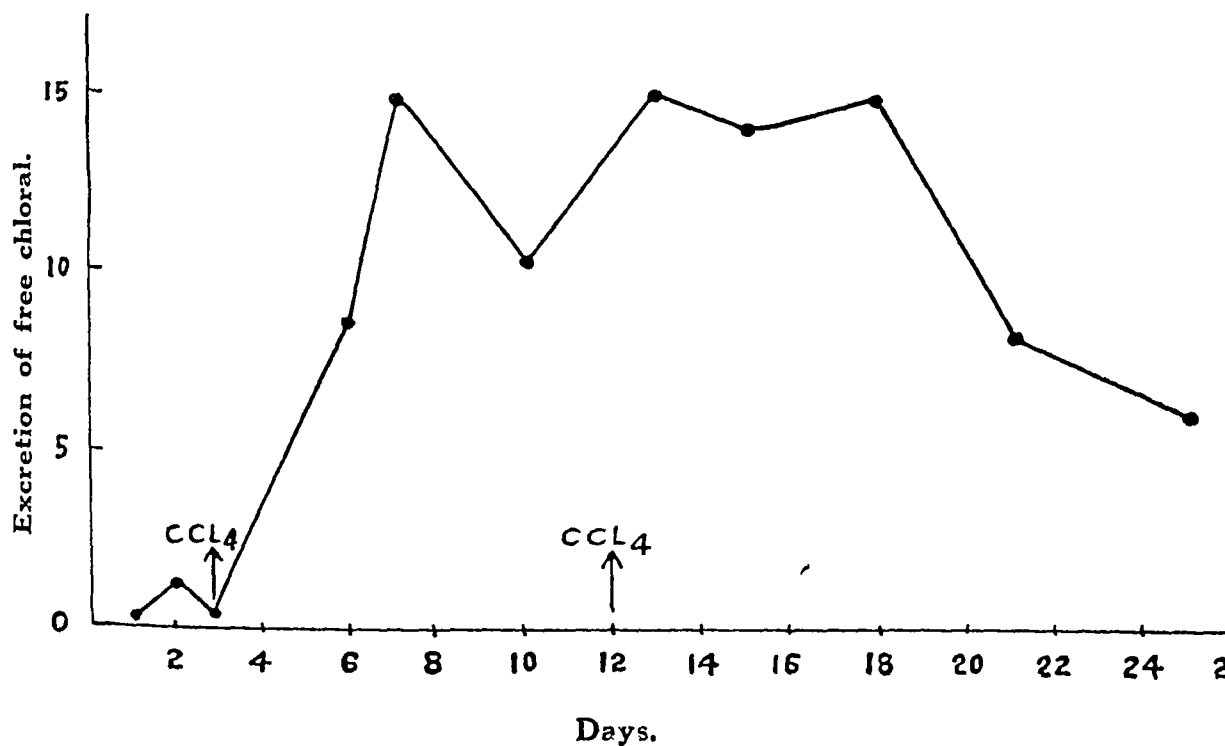
The bottom column of Table II represents the data in 2 dogs (Nos. 1 and 2) where a chronic cirrhosis and liver injury was brought about and maintained during the period of experimentation by intermittent administration of hepatoxic doses of carbon tetrachloride. A dozen experiments were performed in these two animals, each time chloral hydrate (200 mg./kg.) being administered. There has always been a marked increase in free chloral elimination in these animals as evidenced by a markedly deep pink colour, the figures ranging between 12 mg. and 30 mg. in 24-hour specimens (*see* also Graph 2).

GRAPH 2.

Experiments.
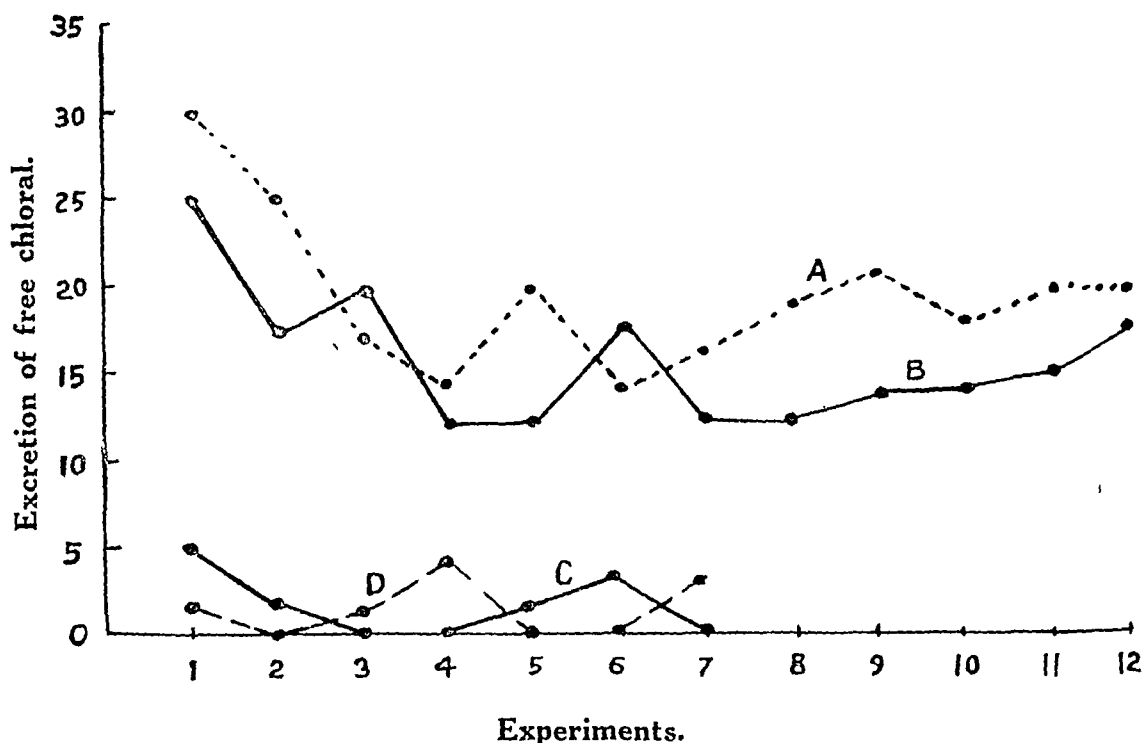
A = dog No. 1, B = dog No. 2, C = dog No. 5, D = dog No. 3.

DISCUSSION.

From the foregoing data, it is evident that under normal conditions in apparently healthy well-fed dogs, oral administration of a dose of chloral hydrate (200 mg./kg.) gives rise to either no appreciable excretion of free chloral or to only very limited amounts of free chloral in 24-hour urine. In six dogs of our series the maximum excretion never exceeded a total figure of 6 mg. in 24-hour urine. In liver-damaged dogs, on the other hand, there is always a significant increase in the amount of free chloral excretion. The increase is seen not only in the dogs which have been exposed to carbon tetrachloride for a long time and whose livers are presumably very severely damaged, but also in those who have been under the influence of the poison for a comparatively short period. Even after one adminis-tration of carbon tetrachloride, the leakage of free chloral becomes evident. This indicates that free chloral elimination may be employed as a criterion of liver damage even in the early stages, an advantage not possessed by many other tests. The test further appears to be a very sensitive one and as low as 0·01 mg. per c.c. (0·001 per cent) of chloral in solution can be quantitatively determined.

The question naturally arises as to whether this criterion can be used as a measure of hepatic efficiency in clinical practice. Theoretically and in the

conditions of our experiments, the test for free chloral after the administration of a known dose of the drug appears to be sound. The writers' experiences are entirely limited to laboratory conditions and unless a fair trial in the clinic is possible, it is too premature to offer any opinion.

A difficulty that may be anticipated in giving this method a trial in clinical practice is the rather large dose of chloral hydrate (200 mg./kg.) employed in our experiments on dogs. If such a large dose of the diagnostic agent has to be employed in eliciting the response, it may be toxic in itself and would obviously be unsuitable. Our dose of chloral hydrate in dogs was chosen as the best suited in the laboratory for giving a significant rise in the excretion of conjugated glucuronic acid which we were estimating in connection with another investigation. Later on, we found that a smaller dose of chloral hydrate (100 mg./kg.) may also serve the purpose. Further work, however, is necessary before it will be possible to suggest the minimum dose of chloral hydrate that may be employed to elicit a satisfactory and measurable response. From certain preliminary experiments that we performed, it may be definitely stated that chloral hydrate is not as toxic as is generally believed and quite a high dose may be given in clinical cases without toxic manifestations to the heart and circulation. Further work on this line is in progress.

SUMMARY.

1. The administration of chloral hydrate in a dose of 200 mg./kg. to apparently normal healthy dogs results either in no urinary elimination of free chloral or in the elimination of free chloral in negligible amounts.

2. In dogs with acute liver damage brought about by the oral administration of one or two hepatoxic doses of carbon tetrachloride, and in dogs with chronic extensive liver damage through the same drug administered intermittently every second or third day for 4 to 5 months, chloral hydrate administration in doses of 200 mg./kg. brings about a significant and well-marked increase in the level of excretion of free chloral.

3. It is suggested that this difference in the level of free chloral elimination may be used as a measure of liver function in human cases. The test is fairly sensitive and promises to be a reliable measure of one of the most important metabolic functions of the organ.

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CAN THE ELIMINATION OF CONJUGATED GLUCURONIC ACID BE USED AS A MEASURE OF HEPATIC EFFICIENCY?

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THE liver has long been regarded as an important detoxifying organ of the body. One of the primary mechanisms through which it protects the body against the constant entry of toxic products by the portal route is its power of 'conjugation' whereby the toxic products are rendered innocuous by coupling with a variety of substances, such as glycine, glucuronic acid, glutamine, cysteine, etc. Of these, glucuronic acid occupies a unique position since it is extensively utilized for this purpose by animals, especially by man. Thus, when certain toxic drugs. e.g., aspirin, chloral hydrate, etc., are introduced into the system, they combine readily with glucuronic acid and are excreted as non-toxic conjugated glucuronides. As this process depends to a large extent on the efficiency of the liver, attempts have naturally been made from time to time to develop hepatic-efficiency tests based on this 'glucuronic function' of the organ. Van Dooren and Destrée (1924) suggested a liver-function test by giving 1 g. of camphor by the mouth and testing the urine for conjugated glucuronide by Grimbert-Bernier's colour reaction. Beaumont and Dodds (1931) described a similar test by administration of aspirin. However, no satisfactory clinical application was made of the principle underlying this test, probably owing to the fact that the tests for detection of conjugated glucuronides in urine available to these workers were only qualitative and not altogether reliable. Considerable doubt also existed (Quick, 1927) as to whether the liver was the only organ responsible for detoxication or whether other organs, as the kidney for example, might take part in the process.

During the last few years interest in the study of the detoxication mechanisms and the functional efficiency of the liver under experimental conditions has been

renewed. Evidence had been secured that the production of glucuronic acid is influenced by liver injury or liver disease (Nishimura, 1935 ; Boku and Kin, 1931 ; Nazarjanz, 1931, 1934 ; Persova, 1930). Convincing experiments have been reported by Hemingway *et al.* (1934) that the conjugation of drugs, such as aspirin, chloral hydrate, menthol, and borneol, with glucuronic acid takes place mainly, if not entirely, in the liver. Salt (1935), after detailed investigation, has proposed a new modification of Tollen's naphthoresorcinol test by which the previous difficulties and disadvantages attending the determination of conjugated glucuronide in the urine could be overcome. In view of the importance of determining the de-toxicating efficiency of the liver in clinical medicine, it was thought profitable to re-investigate the problem in the light of the modern developments, and to see whether any definite correlation could be found between different degrees of liver damage and the rate of excretion of conjugated glucuronic acid.

METHODS.

Animals and their treatments.—Rabbits and dogs were used as our experimental animals. A group of 12 young healthy rabbits weighing on the average 1 kg. to 1·5 kg. and 4 dogs weighing 4·5 kg. to 6·5 kg. were chosen and were placed under identical conditions on a standard diet (consisting of green grass, cabbage or lettuce, soaked peas, and carrots, for rabbits ; and cooked meat, rice, and bread, for the dogs) in metabolism cages. Water was allowed *ad lib.* Arrangements were made to collect 24-hour urine samples daily, only female dogs being used to prevent urine wastage. A week was allowed for the animals to get accustomed to the laboratory conditions and to the diet before experiments were started. During this period, the normal level of day-to-day glucuronic-acid elimination was determined both in the rabbits as well as in the dogs. The animals were then treated in the following manner :—

(1) Chloral hydrate, in gradually increasing doses (starting with 50 mg./kg.), was administered by means of a stomach tube to these animals until a dose was attained at which an appreciable increase in the urinary excretion of conjugated glucuronic acid was observed. (This dose has been termed, for the sake of convenience, the ' effective dose '.) Salt (*loc. cit.*) suggested the use of aspirin on the ground of its being the least toxic of the glucuronogenic drugs. Chloral hydrate was chosen in our experiments partly because we were interested in the pharmacology and toxicology of the drug and partly because we were convinced from previous experiments (unpublished) that chloral hydrate was certainly not as toxic as was usually believed. Further, its hypnotic action was helpful in the study of its blood clearance which we had also undertaken. By repeated trials, a dose varying from 250 mg. to 450 mg. per kilo body-weight was found to bring about an increased excretion in the rabbits. On the other hand, dogs required very much less, a dose of 150 mg. to 200 mg. per kilo being usually sufficient. Individual variations in regard to their response to administered chloral hydrate were much less marked in the dogs than in the rabbits.

(2) Experimental liver damage was then brought about in these animals by the ingestion of slowly increasing doses of carbon tetrachloride (chemically pure). Gardner *et al.* (1925) had found that fatty infiltration and necrosis resembling the liver changes in acute yellow atrophy and chloroform poisoning could be produced

within 24 to 48 hours by doses of carbon tetrachloride as low as 0·176 c.c. to 0·25 c.c. per kg. We therefore started with a higher dose of 0·5 c.c. per kilo which was sure to cause liver damage. In some experiments, this dose was increased to 1·0 c.c. or 1·5 c.c. per kilo. Any attempt to introduce a larger dose (e.g., 2 c.c. per kilo) was usually unsuccessful and attended with fatal results. As our idea was to produce a chronic liver damage and as the liver tissue had been shown to possess a tremendous capacity for quick regeneration, the dose of carbon tetrachloride was repeated every 2nd or 3rd day. Lamson and Wing (1926) poisoned dogs with 0·25 c.c. to 4·0 c.c. per kilo of carbon tetrachloride, given every other day for a period of approximately four months, and showed that under such conditions the liver underwent tremendous necrosis and scar-tissue formation resembling early cirrhosis of the liver. Bollmann and Mann (1932) also showed that long continuous administration of carbon tetrachloride to dog resulted in cirrhosis of the liver. We adopted the method of intermittent administration of carbon tetrachloride to our dogs every alternate day for 18 to 20 weeks. Regularity of administration was generally maintained though sometimes there was a break due to Sundays and holidays intervening. It was not considered necessary to go beyond a dose of 2·0 c.c. to 2·5 c.c. of CCl_4 per kilo as recommended by Chandler and Chopra (1926). This dose was found sufficient to produce the desired liver damage amounting to 50 to 75 per cent of liver destruction. There was often a tendency on the part of dogs to vomit or to be purged when the maximum dose (4 c.c./1 kg. or more) prescribed by Lamson and Wing was tried. This might have vitiated the results as it was difficult to ensure the absorption of the whole dose.

(3) The liver-damaged rabbits and dogs were then given chloral hydrate in doses that were found from previous experiments to be the ' effective dose ' for each animal (250 mg./kg. to 450 mg./kg. in rabbits and 200 mg./kg. in dogs). Chloral hydrate was administered on the day following carbon tetrachloride administration and the determination of conjugated glucuronide carried out the day after. According to Gardner *et al.* (*loc. cit.*) liver damage due to carbon tetrachloride should be sufficiently well marked at this time, and the failure of liver function, if any, should be reflected in the excretion of conjugated glucuronide in urine.

A point worthy of note in this connection is the extreme susceptibility of the rabbit to carbon tetrachloride and chloral hydrate administration. Even under the best of experimental conditions, we lost a few animals within a comparatively short time. Apparently after liver damage, the rabbits could not stand high doses of chloral hydrate for a long time. The dogs, however, tolerated this treatment fairly well. We lost one dog in the middle of our experiments owing to circumstances not directly connected with the technique, but the remaining dogs behaved well, two of them having withstood as many as 60 administrations of carbon tetrachloride and nearly as many administrations of chloral hydrate in about 18 weeks. The dogs, however, lost weight towards the latter part of the experiment and looked listless and apathetic. There was also a marked diminution of their normal appetite. Both the dogs showed a peculiar erythematous condition of the skin attended with diffuse pustular eruptions and loss of hair. This latter effect is probably due to the action of chloral hydrate on cutaneous blood vessels or to its excretion through skin in minute traces.

Analytical methods.—The estimation of conjugated glucuronic acid in urine was carried out by Salt's modification (*loc. cit.*) of Tollen's naphthoresorcinol test. As the quantity of urine voided during 24 hours varied a good deal from animal to animal and from day to day in the same animal, it was found convenient to do the estimations on a quantity of urine which represented a definite proportion (1/15th fraction of total in the rabbit and 1/50th in the dog) of the total amount excreted. In this way, the error due to the multiplication factor was avoided and the quantitative results of day-to-day experiments could be made comparable. The final blue-violet colour of the ethereal extract (pure ether of anæsthetic quality was used throughout) was examined in a Pulfrich Photometer using filter No. S-53 with a wave-length of 530 μ and the result was expressed in terms of the colour absorption (extinction coefficient), which according to Salt (*loc. cit.*) is roughly proportional to the quantity of glucuronic acid excreted. As a specimen of pure glucuronic acid could not be secured, the figures were not corrected and expressed in terms of milligrams of glucuronic acid. However, as the results presented are purely comparative, this is not likely to affect the conclusions drawn from these observations.

Experimental results and comment.

The daily excretion of conjugated glucuronic acid in 24-hour samples of urine and the average rise in its excretion following chloral hydrate are represented in Table I. It will be seen that in the rabbit, the normal glucuronic-acid excretion is rather low and varies only slightly from day to day (range between 1 and 4). In the dog, the average normal excretion is very much higher (range between 40 and 60). The 'effective dose' of chloral hydrate necessary to bring about an increase in the level of the conjugated glucuronic-acid excretion varies considerably in the rabbit, a dose of 250 mg./kg. being sufficient for some, while others require as much as 450 mg./kg. The normal level of glucuronic-acid excretion in the dog, on the other hand, is fairly high and comparatively smaller amounts of chloral hydrate (150 mg. kg. to 200 mg./kg.) are necessary to bring about an increase. These differences are significant and indicate that probably the metabolic processes resulting in the conjugation with glucuronic acid in the system are different in the herbivorous and carnivorous animals.

The excretion of conjugated glucuronic acid following administration of 'effective doses' of chloral hydrate in rabbits poisoned with carbon tetrachloride is shown in the Table II. Only in three animals (Nos. 1, 2, and 3) could carbon tetrachloride be administered in 10 to 11 successive doses, while most of the remaining animals succumbed between 1 and 3 administrations. These rabbits began to show a decrease in weight and appetite after the 4th or 5th administration of carbon tetrachloride and histological examination of the livers secured post mortem revealed in all of them an extensive fatty infiltration and central portal necrosis (*see* Plate XXXIX, figs. 1 and 2). A comparison of the rates of excretion of conjugated glucuronic acid before and after liver damage in these animals reveals three distinct phases: a phase of initial but very temporary decrease, followed by a definite increase of short duration, which again gives place to an ultimate decrease in the conjugated glucuronic-acid excretion. This last phase of decreased excretion is noticeable quite late in the course of the experiments and is almost a terminal event. In the majority

PLATE XXXIX.
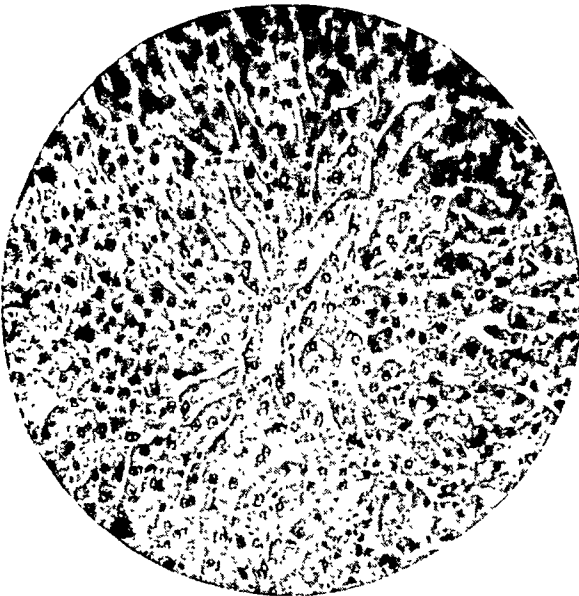
FIG. 1.
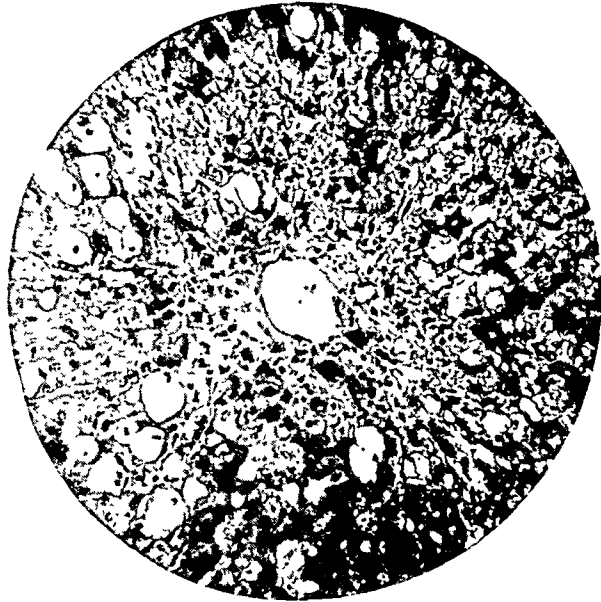
FIG. 2.
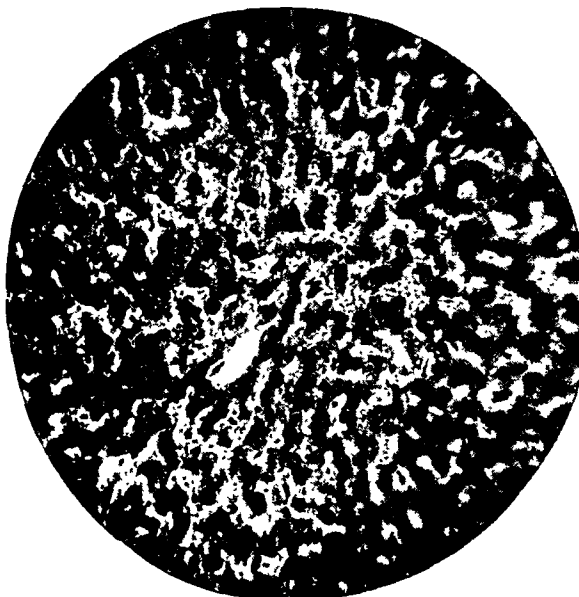
FIG. 3.

FIG. 4.

Figs. 1 and 3.   Microphotograph of sections of normal livers of rabbit and dog, respectively.

Figs. 2 and 4- Microphotograph of sections of livers damaged by oral administration of carbon
tetrachloride.   Note widespread fatty degeneration.   Fig. 2 from rabbit No. 6,
still shows about 30 to 40 per cent of viable liver cells.   Fig. 4 from dog
No. 2, shows a more extensive degeneration and practically the whole liver is
honeycombed with fatty deposits.   In chronic cases of liver damage, strands of
fibrous tissue gradually replacing degenerated cells are also seen (*see* arrow in
Fig. 4).
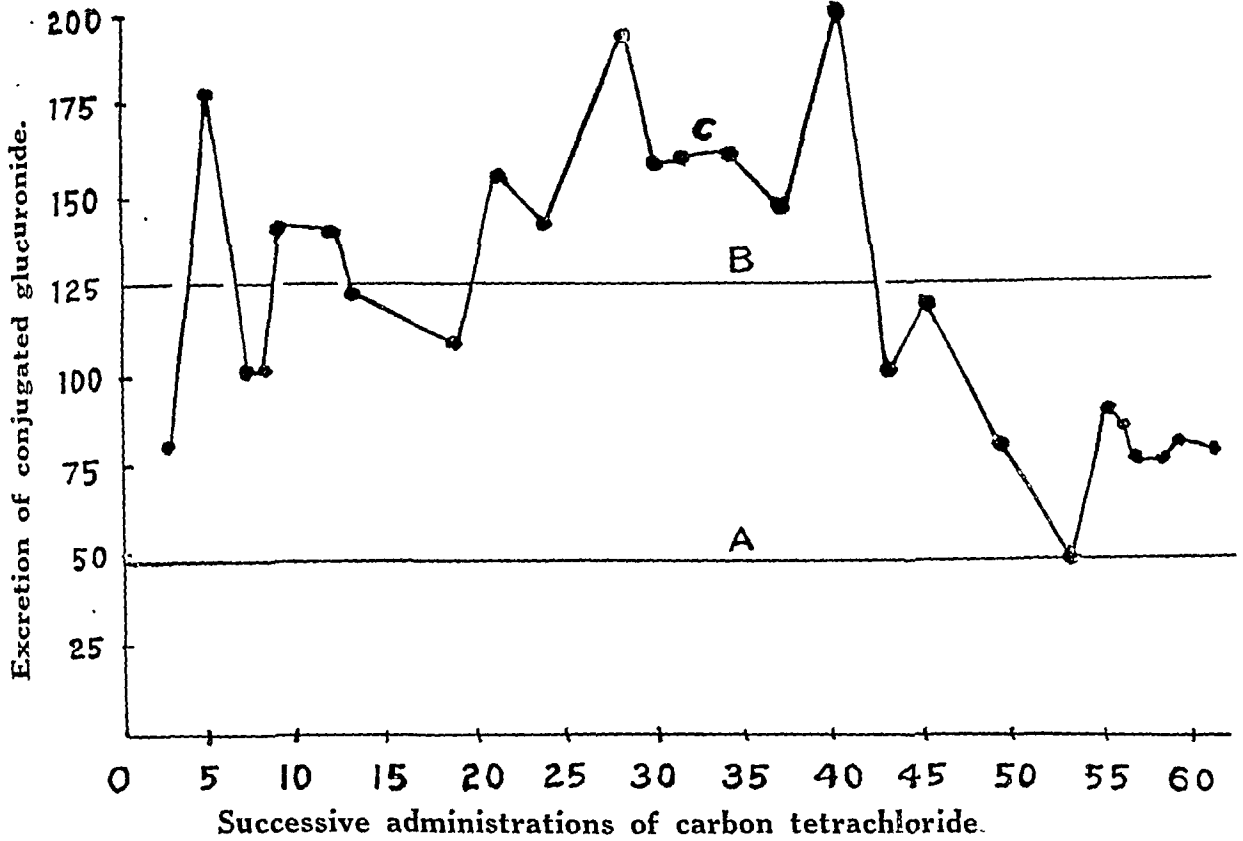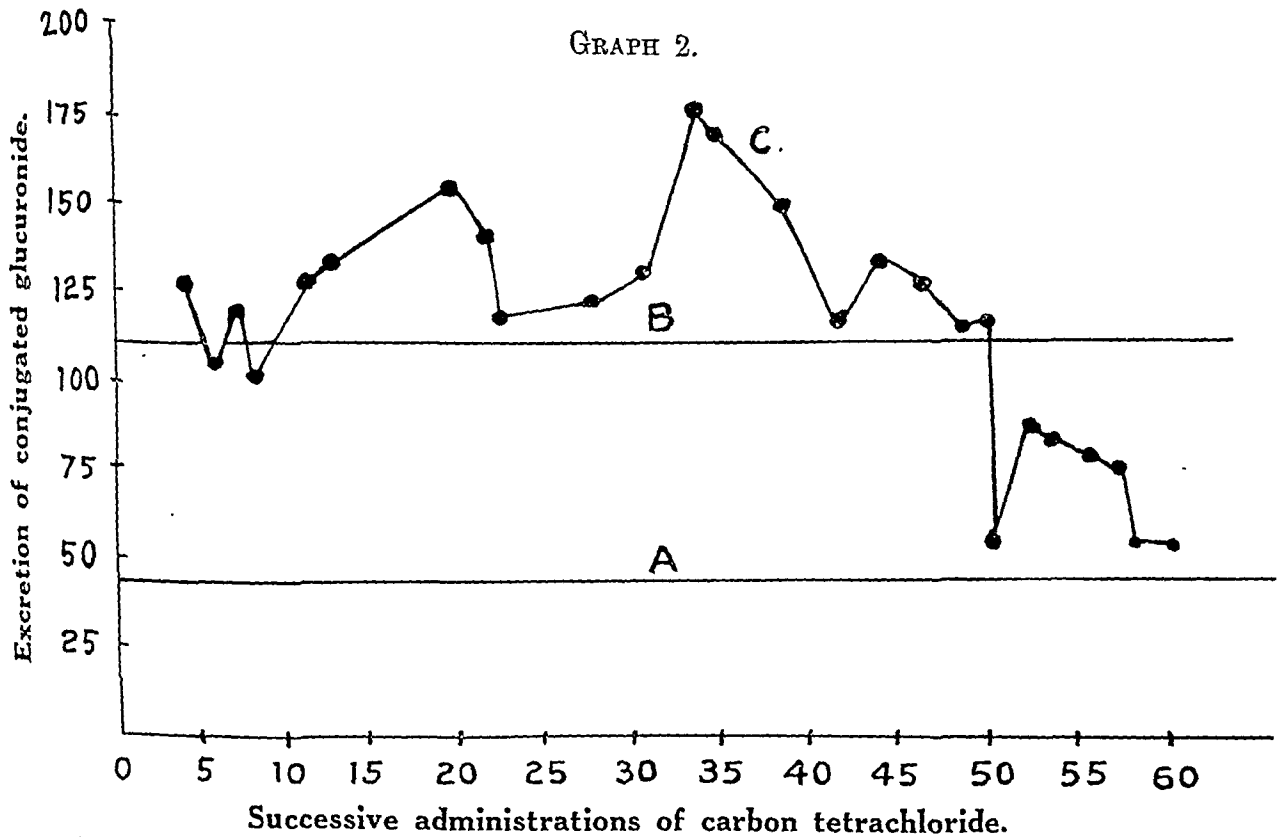
Graphs 1 and 2 demonstrate the excretions of conjugated glucuronide in dogs Nos. 1 and 2, respectively. The straight lines represent the normal daily average excretions (A) and the average daily excretions in normal animals after effective doses of chloral hydrate (B). The curved lines (C) represent the daily excretions following effective doses of chloral hydrate in poisoned animals.

of the remaining animals a phase of increased excretion of glucuronic acid is evident at the beginning which is later followed by a decrease. Only in the case of a few was a phase of decreased excretion not at all apparent, the animals dying within comparatively short time with a rather high level of conjugated glucuronic-acid elimination. It is conceivable that these animals died before the liver damage had progressed to that advanced stage when the decreased excretion would be manifested. This supposition is strongly supported by the histological examinations of the livers of two rabbits (Nos. 7 and 16) which showed only hydropic changes, poor staining properties, and a fatty infiltration of a very minor character without any demonstrable necrosis around portal vein, in sharp contrast to the other rabbits which withstood repeated doses of carbon tetrachloride and chloral hydrate.

Table III summarizes the findings in the three dogs where satisfactory experiments could be performed. Of these, two dogs were brought to a stage of chronic liver damage resembling early cirrhosis of the liver by intermittent administrations of as many as 60 doses of carbon tetrachloride (*See* Plate XXXIX, figs. 3 and 4). The effects on the conjugated glucuronic-acid excretion following the administration of 200 mg./kg. of chloral hydrate at various stages of liver damage were observed in these animals regularly and the data are represented in Graphs 1 and 2. In both these, an initial phase of irregular excretion (decrease or increase) was followed by a well-marked increase. This increase was maintained for a fairly long period though day-to-day variations in the level of excretion was not uncommon. After 40 to 50 intermittent administrations of carbon tetrachloride, a phase of decreased excretion became evident and this decrease was apparently maintained up to the end of the period of experimentation. In dog No. 4, where only six doses of carbon tetrachloride were administered, a definite increase was seen from the beginning.

DISCUSSION.

The results of our experiments bring out several points which need further comment. One interesting observation is the difference in the rabbit and the dog of the normal level of glucuronic-acid excretion and of the ' effective dose ' of chloral hydrate required to cause a significant increase in the excretion of conjugated glucuronic acid. The rabbit has a very low output and requires a comparatively higher dose of a glucuronogenic drug to produce an increase in the elimination of conjugated glucuronic acid. The question therefore naturally arises as to whether the process of detoxication of chloral hydrate or similar drugs is essentially different in the two animals. Detoxication, which consists essentially in the destruction of a toxic compound or in changing a physiologically active chemical group to one which is less so, is brought about by one or other of the processes of oxidation, reduction, or conjugation. It is possible that in the rabbit the processes of oxidation and reduction may play a more important part than conjugation, whereas in the dog the reverse may be the case. This speculation gains support from the statement of Quick (1937) that some amount of glucuronogenic drugs may be destroyed in the body by oxidation. Pryde and Williams (1936) corroborating the earlier work of Quick (1928) found that in man, only about 80 per cent ingested borneol is excreted as borneolglucuronide, the rest being oxidized. Miller *et al.* (1933) suggest that the

dog possesses a store of glucuronic acid or can synthesize it from carbohydrates or amino-acids. In the case of the rabbit, Miller and Connor (1933) have shown that this animal is unable to synthesize glucuronic acid from carbohydrates or amino-acid (or at least performs these synthesis very slowly), but that it can utilize glucuronic acid to a certain extent from its food supply. These considerations offer a fairly satisfactory explanation of the difference in the glucuronic-acid conjugation mechanisms in the two species of animals.

Another point of considerable importance and significance is the finding that at certain stages of liver damage due to intermittently repeated ingestion of toxic doses of carbon tetrachloride, the administration of a glucuronogenic drug such as chloral hydrate may result in an increased excretion of conjugated glucuronic acid. Theoretically one would expect under such circumstances a diminution of conjugated glucuronic-acid excretion owing to the failure of the poisoned liver cells to elaborate glucuronic acid from the carbohydrates or from the glucogenetic amino-acids. Persova (*loc. cit.*) found a diminution of mentholglucuronide after administration of menthol to dogs whose livers were poisoned with arsenic, but as the details of his experiments are not available, it is difficult to compare the results. In three rabbits and two dogs in which we succeeded in bringing about a chronic liver damage associated with extreme fatty infiltration, central necrosis and proliferation of fibrous tissue, we failed to elicit a response tending towards diminution in the rate of excretion of conjugated glucuronic acid during the early stages of liver damage. Both in the rabbits and in the dogs, figures occasionally appeared which were lower than the average normal rate of excretion, but, in general, it may be stated that under the conditions of our experiments, a tendency towards a phase of increased glucuronic-acid excretion was distinctly evident. In a few rabbits and in one dog where only two to four administrations of carbon tetrachloride were possible, and where there was a presumption that the liver damage was recent and not excessively progressive, the response to chloral hydrate was positive rather than negative. When, however, there was reason to believe that the liver damage was excessive and long-standing, a phase of decrease as reported by Persova (*loc. cit.*), Nishimura (*loc. cit.*), and others, was evident. A pertinent question in this connection is how this increased excretion is brought about. It is difficult to hazard a suggestion until more is known about glucuronic-acid metabolism in the animal organism. One explanation may be that hepatic cells when slightly damaged by a comparatively small dose of a toxic drug, make a great effort towards recovery resulting in a hyperactive state of the organ as a whole, or those functions of the organ (e.g., glucuronic function) which have to be mobilized in an effort to deal with the poison. An excessive production of glucuronic acid may result under such circumstances. Another explanation and a more probable one is suggested by the work of Pryde and Williams (*loc. cit.*) that a glucuronogenic drug like borneol is only partially conjugated in the body to form borneolglucuronide. Quick (1928) in an earlier work reported that menthol as well as mentholglucuronide was almost completely oxidized by the dog. In the normal detoxication mechanism therefore it may be assumed that there is a balance between the various processes of oxidation, reduction and conjugation. In early liver damage this balance may be upset, resulting in a preponderance of one and a suppression of the others. An increased excretion of conjugated glucuronic acid under such conditions may not be a true measure of the amount synthesized but rather of the amount which has escaped oxidation.

SUMMARY AND CONCLUSIONS.

1. The urinary elimination of conjugated glucuronic acid by rabbits and dogs kept under standard conditions has been estimated before and after the administration of a glucuronogenic drug (e.g., chloral hydrate), employing an improved and comparatively recent technique. The daily level of conjugated glucuronic-acid excretion varies in the two animals but an 'effective' dose causes in each a distinct rise in the conjugated glucuronic-acid excretion.

2. An attempt was made to bring about chronic liver damage in these animals by intermittent administration of toxic doses of carbon tetrachloride. Rabbits did not stand such treatment well but dogs tolerated it sufficiently to enable long-term observations possible.

3. Effective doses of chloral hydrate were administered to these liver-damaged animals during various periods and observations were made of the effects on the rate of excretion of conjugated glucuronic acid under identical conditions.

4. During the early stages of liver injury, there is usually a tendency both in the rabbits and in the dogs towards an increased excretion of glucuronic acid, though sometimes the rate of glucuronic-acid excretion is quite irregular. During the late stages of liver damage when the microscopic changes in the cells are well advanced, there is a definite decrease in the conjugated glucuronic-acid excretion. The first observation is contrary to accepted theoretical ideas and previous findings. An explanation of this phenomenon has been offered.

5. In view of the fact that the rate of excretion of conjugated glucuronic acid under various conditions of experimental liver damage does not usually follow a regular course at least in the early stages, the employment of this criterion as a measure of liver function does not appear to be promising at present. Further work to elucidate the nature and cause of this increase in glucuronic-acid excretion is called for.

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TABLE I.

Showing normal conjugated glucuronide excretion in rabbits and dogs and how it is influenced by chloral-hydrate administration.   The figures represent photometric measurements.

	Animal number.	Sex.	Weight in kg.	* Excretion in 24 hours. Number of experiments.					† Excretion after chloral hydrate in ' effective dose ' mg. per kg.				
				1	2	3	4	5	150	200	250	350	450
Rabbits.	1	M	1·3	1·1	1·6	2·2	2·9	3·0	..	..	10·3	..	..
	2	M	1·29	1·1	1·6	1·8	2·1	3·0	..	..	8·5	..	..
	5	M	1·63	1·5	1·7	2·1	2·6	3·1	..	..	10·5	..	..
	6	M	1·53	1·5	1·7	2·1	3·1	3·8	..	..	7·5	..	..
	7	M·	1·28	1·3	1·4	1·5	2·1	3·9	..	..	3·9	2·1	10·8
	8	M	1·33	1·5	1·5	2·1	3·0	3·9	..	..	1·8	2·0	12·0
	9	M	1·15	1·7	1·9	2·3	3·2	3·7	..	..	1·9	5·0	..
	12	M·	1·34	2·1	2·4	3·0	3·2	3·5	..	..	2·1	27·0	..
	14	F	1·39	1·2	2·1	3·2	..	..	..	..	..	48·0	..
	15	F	1·14	1·3	1·3	1·7	3·5	3·9	..	..	..	39·0	..
	16	F	1·43	2·1	2·7	3·1	..	..	..	..	2·7	5·1	..
	18	F	1·26	2·2	2·7	3·5	..	..	..	..	3·5	7·5	..
Dogs	1	F	4·76	40	43	44	54	58	80	125	..	..	..
	2	F	4·90	34	35	43	48	50	75	110	..	..	..
	3	F	6·40	40	45	49	55	51	..	115	..	..	..
	4	F	5·40	30	34	35	40	·.	..	90	..	..	..

*Figures arranged in increasing order to show the range of values.
†Average of three determinations in each case.

TABLE II.

Showing the excretion of conjugated glucuronide in rabbits following the administration of 'effective doses' of chloral hydrate after the livers were damaged with carbon tetrachloride.

Figures as in Table I.

RABBIT NUMBER :—	1	2	5	6	7	8	9	12	14	15	16	18
Excretion with 'effective dose' of chloral hydrate, mg./kg. :—	10·3	8·5	10·5	7·5	10·8	12·0	5·0	27·0	48·0	39·0	5·1	7·5
1	..	..	..	..	..	..	9·0	37·2	56·3	39·0	18·6	..
2	..	..	..	..	..	18·0	..	*	42·0	28·8	30·0	9·0
3	..	..	..	..	16·0	23·0	4·5	..	*	*	21·6	*
4	7·5	7·1	..	2·2	*	11·4	*	..	..	..	*	..
5	..	..	8·1	1·0	..	4·5	..	..	..	..	..	..
6	8·7	4·8	..	..	..	*	..	..	..	..	..	..
7	..	..	1·3*	18·9	..	..	..	..	..	..	..	..
8	16·6	10·5	..	20·6	..	..	..	..	..	..	..	..
9	..	10·2	..	..	..	..	..	..	..	..	..	..
10	7·5	..	..	..	..	..	..	..	..	..	..	..
11	*	3·8*	..	3·0*	..	..	..	..	..	..	..	..

(Left margin label: Number of administration of CCl₄.)

Dose of CCl₄ administered :—

Nos. 5, 14, and 18   1   c.c./kg.

Nos. 7, 8, 9, 12, 15 and 16 ..   ..   1·5   c.c./kg.

Nos. 1, 2, and 6   1   c.c./kg. first five administrations ; 1·5 c.c./kg. following six administrations.

*Indicates the point where the animal died.

TABLE III.

Showing the excretion of conjugated glucuronide in dogs following the administrations of 'effective doses' of chloral hydrate after the livers were damaged with CCl_4.

Figures represent photometric measurements as in Table I.

Dog number:—	1	2	4	Dog number:—	1	2	4
Excretion with 'effective dose' of chloral hydrate.	125	110	90	Excretion with 'effective dose' of chloral hydrate.	125	110	90
1	..	..	80	33	..	176	..
2	..	..	160	34	160	169	..
3	80	..	..	37	144	..	..
4	..	127	145	38	..	150	..
5	177	..	..	40	200	..	..
6	..	104	130	41	..	116	..
7	100	121	..	43	100	134	..
8	100	100	..	45	120	..	..
9	140	..	..	46	..	126	..
11	..	127	..	48	..	114	..
12	137	132	..	49	80	117	..
13	122	..	..	50	..	54	..
19	108	160	..	52	..	87	..
21	154	140	..	53	48	85	..
22	..	117	..	55	90	80	..
24	140	..	..	56	84	..	..
27	..	123	..	57	75	75	..
28	194	..	..	58	75	..	..
30	160	130	..	59	80	..	..
31	160	..	..				

Left column label: Number of administration of CCl_4.
Right column label: Number of administration of CCl_4.

Dose of CCl_4 administered:—

Nos. 1 and 2 .. .. .. 0·25 c.c./kg. first eight administrations; 0·5 c.c./kg. following twenty-five administrations; 1 c.c./kg. next fifteen administrations; 2 c.c./kg. last ten administrations.

No. 4 .. .. .. 2 c.c./kg. first six administrations.

LEAD-CONTENT OF HUMAN HAIR.

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IT has been definitely established that lead is present in normal human tissues in variable quantities. The difference in dietaries and in the conditions of living accounts for individual variation and also for variation in different nationalities. Information about the lead-content of human tissues, excreta, and foodstuffs, has been rendered available by several workers in this line but nothing about lead in hair is found in the current literature on this subject.

In the course of an investigation about the lead-content of normal tissues of Indians a few samples of hair were found to contain very large quantities of lead (Bagchi *et al.*, 1939). In fact the quantity is much larger than what is obtained in bones and teeth which are regarded as lead depôts in the human system. As hair is a dead tissue there is no possibility of any lead of hair being returned to the general circulation in a soluble form for subsequent elimination through the usual channels as happens in the case of bones (Leschke, 1934). The amount of lead stored in hair appears to be the amount eliminated from the system. Hair and hair-follicles seem to function as excretory apparatus for lead and possibly for other metals.

About 200 samples of hair from apparently healthy individuals of both sexes, and of various ages from amongst the Europeans, Anglo-Indians, and Indians, were collected and examined. Necessary steps were taken to eliminate all possible contaminations with extraneous lead compounds. The dithizone method of Lynch, Slater and Osler (1934), slightly modified to suit our requirements, was employed

for determination of lead and the method was found quite satisfactory for purposes of this investigation as indicated by results of duplicate analyses and by recovery of added quantities of lead. In all cases giving unusually high or low figures, the experiments were repeated either to confirm or to discard the first result. The specimens giving very high figures were looked upon with suspicion and experiments after repeating the processes of cleaning, were performed in duplicate. In most cases, sufficient material was available for checking the results in various ways.

A large quantity of hair (about one pound) obtained from about thirty men (all Indians), collected from the dusty floor of a hair-cutting saloon and carefully mixed together, formed the stock material for our experiments regarding the efficiency of different methods of cleaning the hair. The first experiment for determination of the quantity of lead in the dirty sample (without cleaning it in any way) gave 52 mg. of lead per kilo. It was then treated, consecutively, with hot soap-solution, ether, hot dilute caustic alkali, water, alcohol, and ether, and lead was determined for the second time and the result was 47·7 mg. The sample was further treated with caustic alkali, hot dilute hydrochloric acid, water, ammonium acetate, alcohol, and ether, and the third determination showed no further reduction in the amount of lead. Hair of women giving very high lead figures and having definite history of abnormal lead exposure, was also treated in the same way and similar results obtained in such cases, proved the efficiency of the technique. The first method was then considered suitable for complete removal of lead contamination and was adopted throughout this investigation. Attempts to reduce the number of steps and simplify the method did not prove successful.

The weight of hair taken for each determination was about two grammes and in some cases, especially in children, it was considerably less. The latter cases did not provide sufficient material for duplicate experiments and the fallacies, if any, could not be eliminated. The results have been shown in the tables in milligrams of lead (as Pb) per kilo of hair which is the same as part per million (p. p. m.).

DISCUSSION.

The tables show that the lead-content of hair of individuals varies very widely. The lead-intake in food cannot account for this wide divergence which at first sight appears to be an extremely unlikely phenomenon. Professor Kehoe of the University of Cincinnati, suggested, in a private communication, the possibility of presence of lead-compounds as contamination in hair. Careful and painstaking methods of cleansing of hair already described may, for all intents and purposes, be considered sufficient to preclude any such possibility. The other metals like copper, zinc, manganese, etc., have also been detected in hair and the determination of their quantities made in course of another investigation on this line (now in progress) also shows similar divergence. As the contamination of hair with salts of nickel, cobalt zinc, manganese, etc., is not at all a probable factor, especially in Indian villages and towns which are extremely backward industrially, the cause for such wide variations is to be sought elsewhere. The functions of minor elements in nutrition and the methods of their excretion are yet to be worked out.

The unusually high lead figures in hair of Bengalee Hindu women (*vide* Table II) led us to make a thorough search for the purpose of finding out the sources of

exposure. The common articles of diet taken by the Bengalees, the cooking utensils (viz., aluminium and brass ware, copper ware tinned inside, enamelled ware, cheap porcelain, etc.) and scented and medicated hair oils were collected from various sources, specially from those families from which hair with high lead-content was obtained, but no lead in appreciable amount could be detected. Subsequently, the source was traced to vermilion which is used by Hindu women on the scalp (as a thin linear mark about $\frac{1}{2}$ to $1\frac{1}{2}$ inch on the anterior end of the hair-parting to indicate unwidowed married life) as an important article of daily toilet. Pure vermilion is red sulphide of mercury imported from China but it is not available everywhere and a cheaper quality consisting of red lead mixed with a red synthetic dye and scented, has therefore been popular among all sections of the community. Vegetable hair oils (coco-nut, sesame, or rape seed oil—scented and unscented) are also used before or after bath and are believed to help the growth of hair and to impart the characteristic gloss to black hair so common among Bengalee women. The use of hair oil helps the absorption of lead through the scalp just in the same way as 'greasy paints and cosmetics containing lead are absorbed through the skin' (Monier-Williams, 1938). Red lead carelessly used may also result in its absorption through the respiratory and alimentary tracts. The fatty acids of vegetable oils also combine with lead compounds and form lead soap on the scalp. The question, therefore, arises : Do the lead soap and red lead stick to hair and scalp in such a way that they cannot be got rid of and do they account for the high lead figures and their wide divergence in individual cases ? The lengthy process of cleansing of hair as described before has been proved experimentally on various specimens of hair to be sufficiently reliable for getting rid of lead contamination and any suggestion for the presence of such contamination is no longer tenable.

Another important observation lends an additional weight to our views. The cleansing of scalp where vermilion is applied is obviously more difficult but specimens of scalp which were examined in this connection gave much too low figures. The sample of hair giving 508 mg. of lead was obtained along with the scalp, as a necropsy material, from a Hindu woman (No. 51 in Table I) from the police morgue. The scalp was found to contain only 1·2 mg. of lead although it could not be submitted exactly to the same lengthy process of cleaning as was done in the case of her hair and it may be noted that the minimum amount of lead which was found in the scalp of a man (not using vermilion) was only 1·0 mg. Similarly, specimens of skin which are equally unsuitable for thorough cleansing, never gave more than 0·5 mg. of lead (Bagchi *et al., loc. cit.*) in spite of the fact that skin is exposed to lead contamination to the same extent.

It is observed that women having large amounts of lead in their hair, also give abnormally high figures of lead in their urine and fæces (*vide* Table IV). For instance, one with 502 mg. of lead in her hair, gave 1·05 mg. in fæces and 0·059 mg. in her urine ; these figures are about ten times the normal for Indians (Bagchi and Ganguly, 1937). It may, therefore, be inferred that red lead is absorbed through the scalp and thrown into the general circulation for elimination through the usual channels but most of it appears to be transferred to the scalp and dumped in hair and thus eliminated from the system.

The figures in Table II show that the average lead-content of hair of the male population of different provinces of India, except Bengal, is practically of the same

general magnitude as that of an average European. The lead-content of hair of both sexes among Europeans (resident in Calcutta) does not show much difference, while among Indians, especially the Bengalee Hindus, the difference is enormous. The Bengalee Mohammedan males, on the other hand, show decidedly larger quantity of lead in comparison with the Hindus. The source of lead, to account for the high lead figures in this community, could not be traced. The question is, however, under consideration.

The relation between lead-content and colour of hair appears to be a definite one. The finest black hair of ladies of respectable families usually contains very large quantities of lead, while grey hair of old men and women gives the minimum figures. Hair with brown, auburn or golden brown and similar other shades of colour also gives very low figures (*vide* Table III). It seems that lead has something to do with the production of pigments which characterize hair of different nationalities. It is not known in which form lead is present in hair. The amounts of sulphur, chlorine, nitrogen, organic matter, etc., determined in samples of hair showing extreme variations in lead-content do not indicate any definite relation existing between lead and those substances. The presence of a large number of metals, viz., iron, copper, nickel, cobalt, zinc, manganese, etc., in hair in large quantities has so far baffled all our attempts to find out the actual nature of the compound which lead forms in hair. Phosphorus is the only element which appears to claim some relation with lead in hair.

The hair of children contains comparatively smaller amount of lead but in families where there is evidence of abnormal exposure, the children, especially the girls, show high lead figures. For instance, in family I (*vide* Table V), the mother gave 315 mg. and her son gave 60 mg., while her 4-year old daughter gave as much as 277 mg. of lead in her hair ; in family IV, the mother's hair contained 242 mg. and her son's hair contained only 9 mg., but the 3½-year old daughter gave about 75 mg. The members of other families also tell the same tale. Although the vermilion containing red lead is used only by married women in their daily toilet and the risk of exposure being open to all the members of the family, it is the girls who are affected most by this exposure, while the boys escape. The young girls seem to be particularly susceptible to exposure and absorb large quantities of lead.

In good many cases, a mild form of alopecia has been found associated with high lead figures. The 'falling off' of hair is a common complaint among Hindu women. Are they the cause and effect in this cycle of events ? It is known that lead produces marked vascular spasm giving rise to various complications in cases of chronic lead intoxication and it is likely that the spasm taking place in the blood vessels of hair follicles when a certain concentration of lead is reached, would cut off the supply of necessary nourishment to hair and thus cause it to fall off. It will be interesting to study the cause of baldness on this line.

The amount of lead is not always uniformly distributed in a hair. In a specimen of a woman's hair, the distal end was found to contain 66 mg., while the proximal end contained 48 mg. only, and in another specimen the difference was practically negligible. As the excretion of lead through hair is likely to vary from day to day, its composition in its different portions should necessarily vary. This fact is of much medico-legal importance and has been of immense

value in expressing opinion about the period of ingestion of a poison in homicidal cases, as for instance, in slow arsenic poisoning.

Hair, thus, appears to be a dumping ground for all surplus metals which served their purpose, possibly as catalysts, in bringing about various physiological reactions but are no longer required by the system. Their passage through the usual excretory channels is perhaps not conducive to the well-being of the organs concerned.

SUMMARY.

1. Hair contains large amount of lead and in cases of abnormal exposure it may retain as much as 508 mg. per kilo. Black hair of women gives the largest amount, while grey hair of men and women gives the minimum, and brown or golden brown or other shades occupy an intermediate position. Lead may be a likely factor in producing the characteristic pigments of hair in different nationalities.

2. The Hindu married women of Bengal are exposed to abnormal lead risk on account of their using vermilion which is adulterated with red lead and as such the average lead-content of their hair (180 mg.) is much higher than that of Mohammedan women of Bengal (50·4 mg.) who never use vermilion. The average amount of lead (26·7 mg.) of hair of Bengalee Hindus (men) is practically of the same magnitude as that of other peoples including Europeans (20·0 mg. to 22·7 mg.). The Bengalee Mohammedans (men) on the other hand give much higher figure (42·4 mg.)—no source of any abnormal exposure has so far been traced to account for this high figure.

3. Large quantities of lead in urine and fæces of some Hindu men and women (giving very high lead figures in their hair) and other factors indicate that lead is absorbed into the general circulation and then eliminated through hair.

4. The actual composition of the lead compound, i.e., the form in which lead is present in hair, cannot yet be stated definitely. Possibly it is present in combination with phosphorus.

5. The method employed in cleaning hair for getting rid of all possible lead contaminations, leaves no room for any doubt about the actual composition of hair with special reference to lead and possibly other poisonous metals.

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Table I.

Lead-content of hair of different nationalities.

The figures indicate amount of lead in milligrams per kilo of hair or parts per million.

Number.	Name.	Nationality.	Religion and age.	Occupation.	Amount of lead as Pb.	Remarks.
1	Mrs. I. B. ..	Bengalee	H. 45	..	57·0	Distal end 66 mg. Proximal end 48 mg.
2	Mr. J. S. ..	,, .	H. 32	Chemist	30·0	....
3	Mr. H. D. G. ..	,,	H. 34	,,	34·0	....
4	Mrs. J. D. ..	Marwari	H. 22	..	30·5	....
5	Mrs.—— ..	Anglo-Indian	C. 35	..	16·5	....
6	Mrs.—— ..	European	C. 30	..	17·0	....
7	Mrs.—— ..	,,	C. 48	..	33·0	....
8	Miss—— ..	,,	C. 20	Student	16·5	Blonde.
9	Miss—— ..	,,	C. 14	,,	21·0	Golden brown.
10	Master S. G. ..	Bengalee	H. 7	..	9·0	....
11	Master K. B. ..	,,	H. 9	Student	24·0	....
12	Master A. B. ..	,,	H. 11	,,	15·0	....
13	Master S. B. ..	,,	H. 13	,,	16·5	....
14	Mr. A. B. ..	,,	H. 15	,,	16·5	....
15	Mr. N. B. ..	,,	H. 18	,,	27·0	....
16	Mr. K. B. ..	,,	H. 50	Chemist	27·0	....
17	Mr. G. P. ..	Anglo-Indian	C. 18	Student	15·0	....
18	Mrs.—— ..	Bengalee	H. 65	..	9·0	Grey about 75 per cent.
19	Miss K. M. ..	,,	H. 17	Student	108·0	....
20	Mr. M. R. ..	,,	M. 19	,,	22·5	....
21	Mr. M. A. ..	,,	M. 28	,,	21·0	....
22	Mr. U. H. ..	,,	M. 19	,,	19·5	....
23	Mr. K. K. I. ..	,,	M. 36	Clerk	42·0	....
24	Miss P. M. ..	,,	H. 19	Student	122·0	....

TABLE I—*contd.*

Number.	Name.	Nationality.	Religion and age.	Occupation.	Amount of lead as Pb.	REMARKS.
25	Mrs. K. M. ..	Bengalee	H. 25	..	284·0	....
26	Miss G. M. ..	,,	H. 14	Student	105·0	....
27	Mr. P. P. A. ..	,,	H.	Compositor	50·0	Suspected cases of lead poisoning from Calcutta Medical College Hospital.
28	Mr. N. K. B. ..	,,	H.	,,	132·0	
29	Mr. M. A. ..	,,	M. 26	Student	24·0	....
30	Mr. M. I. ..	,,	M. 32	,,	25·5	....
31	Mr. M. L. ..	,,	M. 28	Doctor	22·5	....
32	Mr. U. K. ..	,,	M. 30	,,	27·0	....
33	Mr. B. ..	European	C. 53	,,	21·0	Grey.
34	Mrs. P. B. ..	Marwari	H.	..	32·5	....
35	Mr. M. H. M. ..	Bengalee	M. 28	Doctor	27·0	....
36	Mr. G. B. ..	European	C.	Clerk	40·5	....
37	Mr. P. D. S. ..	,,	C.	,,	36·0	....
38	Mrs. B. ..	Bengalee	H. 39	..	72·0	....
39	Mr. M. M. ..	,,	H. 49	Doctor	13·5	Grey about 80 per cent.
40	Miss B. S. ..	,,	H. 32	School mistress	222·0	....
41	Mr. R. A. ..	Parsee	Z. 17	Student	10·5	....
42	Mr. P. K. R. ..	Bengalee	H. 19	,,	25·5	....
43	Mr. T. S. ..	,,	H. 28	Clerk	50·0	....
44	Mrs. L. ..	,,	M. 23	..	30·0	Brownish.
45	Mr. S. M. V. ..	Punjabi	H. 36	Smelter	20·0	,,
46	Mr. D. B. ..	Bengalee	H. 28	Typist	24·0	....
47	Mr. B. G. ..	,,	H. 31	Clerk	27·0	....
48	Mr. M. S. ..	,,	M. 19	Student	22·5	....
49	Mr. A. H. ..	,,	M. 27	,,	30·5	....
50	Mr. A. A. ..	,,	M. 23	,,	27·0	....

TABLE I—*contd.*

Number.	Name.	Nationality.	Religion and age.	Occupation.	Amount of lead as Pb.	REMARKS.
51	Mrs.—— ..	Bengalee	H. 25	..	508·0	Died by sulphuric acid poisoning.
52	Mrs. G. S. ..	Anglo-Indian	C. 50	..	30·0	....
53	Miss J. B. A. ..	Marwari	H. 19	Student	54·0	....
54	Miss M. B. ..	Bengalee	H. 15	,,	170·0	....
55	Miss K. B. ..	,,	H. 19	,,	156·6	....
56	Mrs.—— ..	Bhutia	60	Ayah	8·6	Grey.
57	Mr. E. H. ..	Bengalee	M. 32	Clerk	28·5	....
58	Master—— ..	,,	H. 6	..	72·0	....
59	Mrs. H. D. G. ..	,,	H. 30	..	242·0	Alopecia.
60	Mrs. L. D. ..	,,	H. 30	..	302·0	Fine black.
61	Miss S. ..	Parsee	65	..	6·0	Grey.
62	Mr. A. R. C. ..	Bengalee	H. 51	Doctor	16·5	Grey about 75 per cent.
63	Mrs. M. M. ..	,,	H. 40	..	503·3	Alopecia.
64	Miss A. M. ..	,,	H. 22	Student	84·0	....
65	Mrs.—— ..	European	..	..	50·0	....
66	Miss—— ..	,,	15	Student	9·0	Blonde.
67	Miss—— ..	,,	35	..	9·0	Golden brown.
68	Mrs.—— ..	,,	57	..	7·5	Grey.
69	Mrs. S. S. ..	Bengalee	H. 23	..	322·0	Anæmia and alopecia.
70	Mrs. A. K. S. ..	,,	H. 21	..	502·0	No alopecia; fine black.
71	Miss M. B. ..	,,	H. 17	Student	108·0	....
72	Mr.—— ..	,,	M.	..	102·0	....
73	Mrs. B. P. ..	,,	H. 23	..	52·0	....
74	Mrs.—— ..	European	33	..	12·0	Brown.
75	Miss—— ..	,,	25	Typist	12·0	,,
76	Mrs.—— ..	,,	35	..	15·0	,,
77	Mrs.—— ..	,,	42	..	13·5	Blonde.

TABLE I—*contd.*

Number.	Name.		Nationality.	Religion and age.		Occupation.	Amount of lead as Pb.	REMARKS.
78	Mrs. D. A.	..	Bengalee	M.	43	..	69·0	....
79	Miss M. S.	..	Punjabi	Sikh	14	Student	57·0	....
80	Miss T. S.	..	,,		8	,,	69·0	. .
81	Mr. K. S. M.	..	,,		40	Chemist	35·0	....
82	Mr. N. S.	..	,,		35	Typist	45·0	....
83	Mr. N. N. M.	..	Bengalee	H.	54	Zemindar	6·0	Grey.
84	,,	..	,,	H.	54	,,	4·5	Grey (beard).
85	Mrs. J. N. K.	..	,,	M.	34	..	33·0	....
86	Mrs.——	..	,,	M.	25	..	72·0	....
87	Miss K.	..	,.	M.	16	..	37·0	....
88	Mrs. K.	..	,,	M.	30	..	63·0	. . .
89	Mrs. T.	..	,,	M.	24	..	26·3	....
90	Mrs.——	..	,,	M.	40	..	61·5	....
91	Mrs. M. M.	..	,,	M.	50	..	18·0	....
92	Mrs. B.	..	,,	M.	35	..	42·0	....
93	Mrs. B.	..	,,	M.	18	..	60·0	....
94	Mr. S. C. S.	..	,,	H.	50	Doctor	17·1	Grey about 90 per cent.
95	Mrs.——	..	.,	M.	50	..	33·0	,,
96	Mrs.——	..	.,	M.	40	..	62·0	....
97	Mrs.—— .	..	,,	M.	25	..	49·5	....
98	Mrs.——	..	,,	M.	35	..	59·5	....
99	Mrs.——	..	,,	M.	30	..	42·0	....
100	Mrs.——	..	,,	M.	28	..	88·5	....
101	Mrs.——	..	,,	M.	38	..	56·4	....
102	Mrs.——	..	,,	M.	42	..	57·0	....
103	Mrs.——	..	,,	M.	45	..	58·5	....
104	Mrs.——	..	.,	M.	20	..	40·5	....
105	Mr. M. H.	..	,,	M.	20	Student	21·0	....

TABLE I—*contd.*

Number.	Name.	Nationality.	Religion and age.	Occupation.	Amount of lead as Pb.	REMARKS.
106	Miss B. R. ..	Bengalee	H. 4	..	227·0	Fine black.
107	Mrs. B. R. ..	,,	H. 28	..	315·0	,, Alopecia.
108	Baby R. (male) ..	,,	H. 1½	..	60·0	....
109	Mr. B. R. ..	,,	H. 35	Police	19·5	....
110	Mr.—— ..	Bihari	H.	Clerk	20·0	....
111	Mr.—— ..	,,	H.	Police	7·5	....
112	Mr. D. S. N. ..	Madrassi	H. 41	Chemist	8·2	....
113	Mrs. D. S. N. ..	,,	H. 31	..	45·0	....
114	Mr. P. I. R. ..	,,	H.	..	54·0	From Alipore Central Jail.
115	Mr.—— ..	,,	H.	..	30·0	,,
116	Mr. B. S. ..	Nepali	H.	..	37·5	,,
117	Mr. F. M. ..	Punjabi	M.	..	15·0	,,
118	Mr. M. Y. ..	Behari	M.	..	33·0	,,
119	Mr. S. K. S. ..	Oriya	H.	..	31·5	,,
120	Mr. C. N. N. ..	Madrassi	H.	..	32·7	,,
121	Mr. H. A. ..	Bombay	M.	..	60·0	,,
122	Mr. R. E. G. ..	U. P.	Jew	..	39·3	,,
123	Mr. E. E. ..	,,	Jew	..	4·6	,, Grey.
124	Mr. S. S. L. K. ..	,,	H.	..	37·1	,,
125	Mr. K. S. ..	Punjabi	Sikh	..	6·0	,,
126	Mr.—— ..	Madrassi	H.	..	16·0	Brownish.
127	Mr. M. M. ..	Bengalee	M.	..	42·0	From Alipore Central Jail.
128	Mr. N. A. ..	,,	M.	..	78·0	,,
129	Mr. S. A. ..	,,	M.	..	166·0	,,
130	Mr. G. S. ..	,,	M.	..	57·0	,,
131	Mr. A. S. ..	,,	M.	..	40·5	,,
132	Mr. R. R. ..	Madrassi	H.	..	3·2	From Presidency Jail.

TABLE I—*contd.*

Number.	Name.	Nationality.	Religion and age.	Occupation.	Amount of lead as Pb.	REMARKS.
133	Mr. D. D. ..	Madrassi	C.	..	24·0	From Presidency Jail.
134	Mr. B. B. ..	,,	H.	..	3·0	From Presidency Jail. Grey.
135	Mr. R. S. ..	,,	H.	..	33·0	,,
136	Mr. M. N. ..	,,	H.	..	11·1	,,
137	Mr. D. H. D. ..	Anglo-Indian	..	..	22·5	,,
138	Mr. E. W. ..	,,	..	..	6·0	,,
139	Mr. A. J. W. ..	,,	..	..	30·0	,,
140	Mr. S. J. ..	,,	..	..	3·7	,,
141	Mr. B. T. D. ..	Madrassi	..	..	34·5	,,
142	Mr. H. S. ..	Punjabi	Sikh	..	12·0	,,
143	Mr. S. Y. ..	,,	M.	..	4·5	,,
144	Mr. S. S. ..	,,	Sikh	..	41·5	,,
145	Mr. R. S. ..	,,	Sikh	..	3·0	From Presidency Jail. Brown.
146	Mr. R. J. ..	Anglo-Indian	..	..	10·5	,,
147	Mr. A. M. ..	Bombay	M.	..	4·5	From Presidency Jail. Grey.
148	Mr. S. L. ..	,,	H.	..	10·8	,,
149	Mr. P. D. ..	,,	H.	..	12·0	,,
150	Mr. A. K. ..	,,	M.	..	7·5	,,
151	Mr. S. L. ..	,,	H.	..	6·0	,,
152	Mr. M. B. ..	U. P.	H.	..	10·5	,,
153	Mr. G. L. ..	,,	H.	..	15·0	,,
154	Mr. H. M. ..	,,	M.	..	9·0	,,
155	Mr. R. L. T. ..	,,	H.	..	55·0	,,
156	Mr. S. Y. ..	,,	M.	..	7·8	....
157	Mrs. K. ..	Punjabi	Sikh 30	..	10·5	....
158	Mrs. M. D. ..	Bengalee	H. 19	..	126·0	History of abortions. W. R. negative.

TABLE I—*concld.*

Number.	Name.	Nationality.	Religion and age.	Occupation.	Amount of lead as Pb.	REMARKS.
159	Mrs.—— ..	Bengalee	H. 32	..	81·0	....
160	Mrs.—— ..	European	42	..	13·5	Golden brown.
161	Miss H. D. G. ..	Bengalee	H. 4	..	74·8	....
162	Mrs. H. B. ..	,,	H. 24	..	162·0	....
163	Miss B. C. ..	,,	H. 19	Student	100·0	....
164	Miss R. C. ..	,,	H. 14	,,	116·0	....
165	Miss P. C. ..	,,	H. 12	,,	92·0	....
166	Miss A. C. ..	,,	H. 10	,,	104·0	....
167	Miss N. C. ..	,,	H. 8	,,	108·0	....
168	Mr. M. C. G. ..	,,	H. 50	Compositor	13·5	50 per cent grey.
169	Mr. K. P. D. ..	,,	H. 24	Lead worker in a factory.	241·0	....
170	Mr. A. T. D. ..	,,	H. 39	Compositor	36·0	After de-leading treatment.
171	Mixed hair from an Indian hair-cutting saloon.	Bengalee, males.	H. & M.	..	47·7	About thirty specimens mixed together.

TABLE II.

Average lead-content of hair in different nationalities.

The figures indicate amount of lead in milligrams per kilo or parts per millions.

Nationality.	Men.	Women.
Europeans (including Jews and Anglo-Indians)	20·8	18·4
Indians	28·0	114·5
Bengalee Hindu	26·7	180·9
Bengalee Mohammedan	42·4	50·4
Punjabi	20·2	45·5
Madrassi	22·7	..
U. P. and Bihari	21·6	..
Other provincials (Parsee, Marwari, Oriya, etc.) ..	20·0	26·3

TABLE III.

Lead-content of hair of different shades of colour.

Milligrams per kilo or parts per million.

Colour of hair.	Minimum.	Maximum.
Deep black (Bengalee women)	170·0	508·0
Brown, auburn and other shades (Europeans)	9·0	16·5
Grey—containing 0 to 25 per cent of black or brown (Europeans and Indians).	3·0	21·0

TABLE IV.

Lead-content of hair, urine, and fæces of the same individuals.

Milligrams per kilo or parts per million.

Numbers in Table I.	Hair.	Urine.	Fæces.	REMARKS.
28	132·0	0·02	7·8	Suspected lead-poisoning case.
70	502·0	0·059	1·29	Quite healthy with a suckling baby. No lead in her milk.
107	277·0	..	1·05	Unhealthy child.
158	126·0	0·02	.1·04	History of abortions.
169	241·0	0·04	387·1	Lead worker in a factory. Suspected malingerer for workman's compensation.

TABLE V.

Lead-content of hair of members of certain Bengalee Hindu families where vermilion is used. More lead appears to be imbibed by the girls than by the boys in the same household.

Milligrams per kilo or parts per million.

Family.	Numbers in Table I.	Relation.	Age.	Amount of lead.	Vermilion used or not.
I.	109	Husband	35	19·5	Not used.
	107	Wife	28	315·0	Used.
	106	Daughter	4	277·0	Not used.
	108	Son	1½	60·0	,, ,,
II.	39	Husband	49	13·5	Not used.
	63	Wife	40	503·3	Used.
	64	Daughter	22	84·0	Not used.
	24	,,	19	122·0	,, ,,
	19	,,	17	108·0	,, ,,
III.	16	Husband	50	27·0	Not used.
	38	Wife	39	72·0	Used.
	70	Daughter	21	502·0	,,
	71	,,	16	108·0	Not used.
	11	Son	9	24·0	,, ,,
IV.	3	Husband	34	34·0	Not used.
	59	Wife	30	242·0	Used.
	47	Brother	31	27·0	Not used.
	60	Sister	30	302·0	Used.
	10	Son	7	9·0	Not used.
	161	Daughter	3½	74·8	,, ,,

TABLE V—*concld.*

Family.	Numbers in Table I.	Relation.	Age.	Amount of lead.	Vermilion used or not.
V.	25	Mother	28	284·0	Used.
	26	Daughter	12	105·0	Not used.
	15	Nephew	18	27·0	,, ,,
	14	,,	15	16·5	,, ,,
	13	,,	13	16·5	,, ,,
	12	,,	11	15·0	,, ,,
VI.	46	Uncle	28	24·0	Not used.
	162	Niece.	24	162·0	Used.
	163	,,	19	100·0	Not used.
	164	,,	14	116·0	,, ,,
	165	,,	12	92·0	,, ,,
	166	,,	10	104·0	,, ,,
	167	,,	8	108·0	,, ,,

STUDIES ON HÆMOLYSIN OF COBRA VENOM.

Part II.

EFFECT OF DIFFERENT SUBSTANCES ON THE ACTIVITY OF COBRA (*NAJA NAJA*) HÆMOLYSIN.

BY

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(*Inquiry under the Indian Research Fund Association, University College of Science & Technology, Calcutta.*)

RYVOSH (1907) suggested that hæmolysis is of two kinds : the saponin type and the snake venom type. In the first, the cell membrane appears to be dissolved or disrupted and in the second, the cells behave as though disrupted by hypotonic solution. Cells which are resistant to venom hæmolysis are sensitive to saponin type and vice versa.

According to Herrmann and Rohner (1925), however, there are three types of hæmolysis :—

(*a*) Lysis of the lipoid or protein lipoid envelope (adsorption hæmolysis).

(*b*) Disturbance of the isotonic equilibrium (coagulation hæmolysis).

(*c*) Dissolving out of hæmoglobin.

Snake-venom hæmolysis is an ' adsorption hæmolysis '.

The above classification resulted from the study of the mechanism involved in the acceleration or inhibition of hæmolysis by various substances.

Noc (1904) was the first to determine the hæmolytic power of venoms *in vitro*. The hæmolytic power of different venoms was compared by him by determining the time required for complete hæmolysis of 1·0 c.c. of 5 per cent washed corpuscles by 1 mg. of venom in the presence of 0·2 c.c. of inactivated horse serum. This method overlooks the fact that some venom hæmolyse red blood cells of certain groups of animals in the absence of serum, while others produce

hæmolysis only in the presence of inactive serum and lastly hæmolysis by some venom is inhibited by serum. The red blood cells of different species of animals can be divided into three groups according to the inhibiting or accelerating effect of homologous serum on their hæmolysis by cobra venom.

1. Those not hæmolysed either in the presence or absence of homologous serum, e.g., sheep and ox.

2. Those whose hæmolysis by venom do not require the presence of an activator and whose hæmolysis is inhibited by the presence of serum to a greater or less extent, e.g., cat, rabbit, guinea-pig, and human.

3. Those whose hæmolysis is accelerated by the presence of homologous serum, e.g., horse.

With the variation in the dosage of venom to a fixed quantity of red blood cells and homologous serum, difference of behaviour in regard to inhibition or acceleration of hæmolysis by the serum are sometimes displayed.

In this connection it may be mentioned that the hæmolytic power of different venoms varied with different species of red blood corpuscles. Using red blood corpuscles of horse with inactivated horse serum the order of hæmolytic activity of different venoms is as follows: cobra > tiger snake > death-adder > black tiger snake, and lastly comes copper-head venom. While using red blood cells of rabbit and homologous serum the Australian snake venoms are more active than cobra venom.

Until recently no extensive survey was made of the hæmolytic activities of different venoms. Kellaway and Williams (1933) investigated, among other matters, the effect of adding lecithin, the inhibition by serum and the changes due to the absence of ions from the fluid in which the venoms were dissolved and cells suspended.

The present work deals with the influence of certain factors on the hæmolysis of red blood cells of guinea-pig by cobra (*Naja Naja*) venom. The effects studied were those due to variations of the hydrogen-ion concentration, the addition of certain proteins, lipoids, and salts.

EFFECT OF HYDROGEN-ION CONCENTRATION ON HÆMOLYSIS.

Hydrogen-ion concentration has much influence on hæmolysis by snake venoms. *In vivo*, and in systems heavily buffered with serum *in vitro*, this influence is not so important. The major part of the work on hæmolysis with washed red blood corpuscles has lost much of its significance because the hydrogen-ion concentration has not been properly controlled. Holden (1934) studied the effect of change in pH on hæmolysis and much complexity of its action was indicated. He found that variation in hydrogen-ion concentration not only altered the rate of hæmolysis but modified the form of the time-concentration curves. His results revealed the fact that the Australian copper-head venom contains two active agents. One of them has a direct action on red blood cells and most effective at pH 6 and another, which is similar to phosphatidase, acts by the formation of chemical lysins and is

most effective at pH 7·7. The first hæmolyses the red blood cells of rabbit and guinea-pig but not those of the ox and sheep. Its action is inhibited by excess of

GRAPH 1.
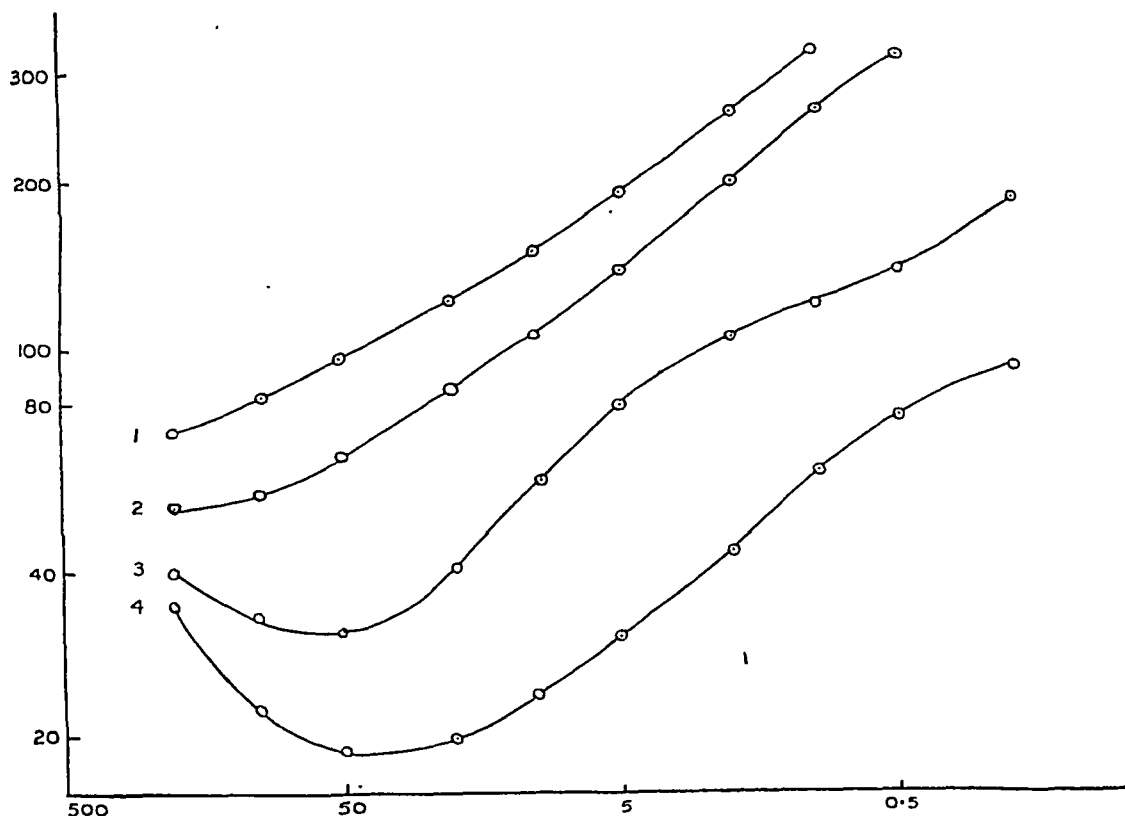
Abscissæ : milligrams of crude cobra venom per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
　　　　　　Both are plotted on a logarithmic scale.

GRAPH 1. Effect of hydrogen-ion concentration on hæmolysis by crude venom.

Curve 1. Acetate buffer in 0·81 per cent NaCl pH 5·6.
　,,　2.　　,,　　　　,,　　　　　,,　　　　,,　pH 6·0.
　,,　3.　　,,　　　　,,　　　　　,,　　　　,,　pH 6·6.
　,,　4.　　,,　　　　,,　　　　　,,　　　　,,　pH 7·6.

venom, and by proteins such as serum albumin. The second active agent hæmolyses all species of red blood cells in the presence of lecithin.

The method employed for determining the hæmolytic activity was that of Ponder (1921) with slight modifications. No extra-cellular lecithin was added to

GRAPH 2.
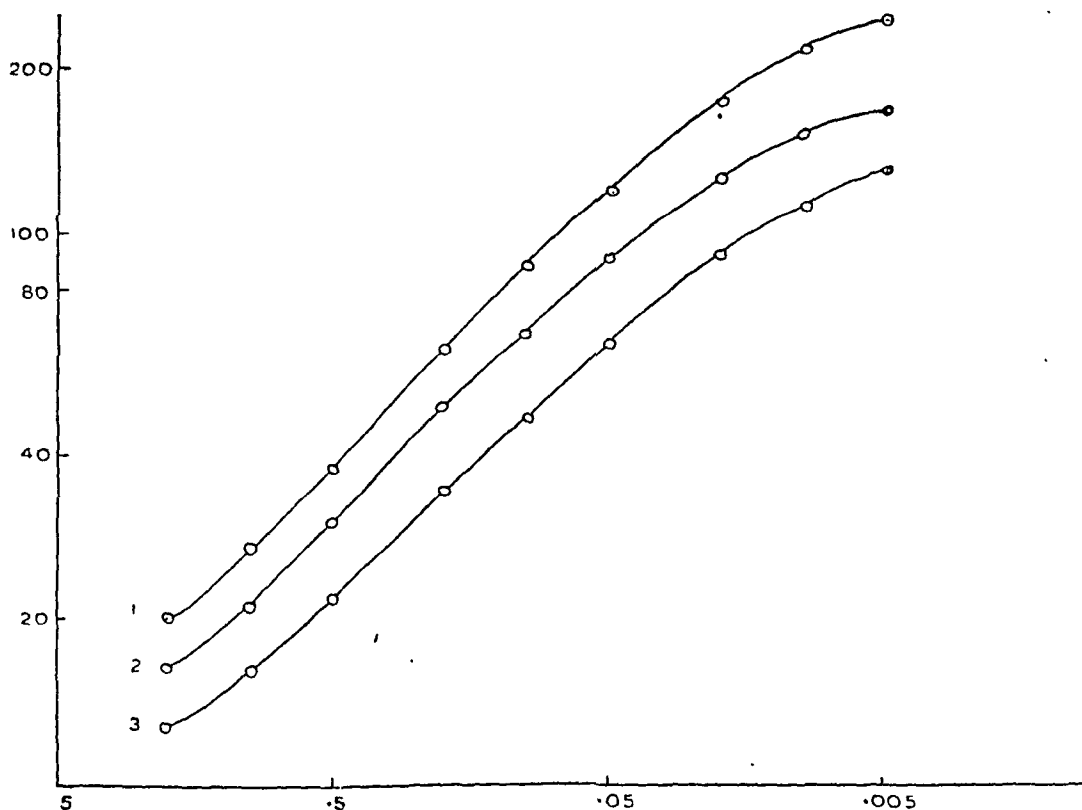
Abscissæ : milligrams of purified hæmolysin per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
          Both are plotted on a logarithmic scale.

GRAPH 2. Effect of hydrogen-ion concentration on hæmolysis by purified hæmolysin.

Curve 1. Acetate buffer in 0·81 per cent NaCl pH 6·0.
  ,,   2.   ,,   ,,   ,,   ,,   pH 6·6.
  ,,   3.   ,,   ,,   ,,   ,,   pH 7·6.

the mixture of venom and red blood cells. The cells were washed as described in the previous paper (De, 1939). As observations were made at different pH the

red blood cells for a particular experiment were washed and suspension made in buffered saline of the same composition as that in which the venom was dissolved. Solutions containing the venom and saline to a total volume of 1·0 c.c. were placed in small tubes. These tubes were then placed in a water-bath at 37°C. Then 0·4 c.c. of 5 per cent suspension of the washed red blood cells of guinea-pig was added and the mixture shaken.

The maximum hæmolytic activity of cobra venom was observed at pH 7·6 (Graph 1) and minimum at pH 5·6 but the maximum activity has been observed by Holden at pH 6·0 and minimum at pH 7·2. It is to be inferred that the 'phosphatidase' is in great excess than the direct hæmolysin in cobra venom. But the form of curve at any particular pH is in both cases the same. At pH 5·6 to pH 6·0 cobra hæmolysin behaved in the same way as copper-head hæmolysin. Time-concentration curves show that the logarithms of the concentrations are proportional to the reciprocal of the logarithms of the times. At more alkaline pH values (6·6, 7·6, and 8·2) the rate of hæmolysis was increased and at high concentrations of venom the curves tended to be flattened with respect to the axis of concentration. In these regions the time of hæmolysis is but little affected by increasing the concentration of venom. The concavity of the curve at high concentration increases as the alkalinity of the medium is increased. This protective effect of large doses of cobra venom on the red blood cells of man was first observed by Stephens and Myers (1898). Kyes (1902) and Kyes and Sachs (1903) observed the inhibition of hæmolysis of red blood cells of rabbit by large doses of venom. Kyes (1910) by means of quantitative relationship between intra-cellular lecithin and amount of venom explained the inhibition caused by an excess of venom. He also showed that excess of venom caused the same inhibition when lecithin was added to non-susceptible varieties of cells. Noguchi (1905), by treating red blood corpuscles of horse with excess (4 per cent) of cobra venom, made them less susceptible to hæmolytic agents such as saponin, hypotonic saline, or lecithin. He thought that a compound of venom with some constituent of the corpuscles was formed under those conditions. In these experiments with purified hæmolysin the inhibiting action noted by Kyes and Noguchi was found to be absent. The results obtained by the author show that at all pH, time-concentration curves plotted logarithmically have a linear relationship. The purified hæmolysin obtained from any one of the two methods described in the previous paper (De, *loc. cit.*) behaved similarly. The curves (Graph 2) are for the purified hæmolysin obtained by the second method.

Effect of Heat on the Hæmolytic Activity of Crude Venom and of Purified Hæmolysin Solution.

Rousseau (1934) observed that heating a solution of cobra venom at 70°C. for 30 minutes and cooling the activity was 94 per cent of the original value as determined by the rate of formation of lyso-lecithin from egg-lecithin. Heating the solution of our cobra venom at 60°C. the activity was not depressed but the hæmolytic activity was slightly increased probably due to the slight destruction of the hæmolysin inhibitor. At 75°C. the activity was reduced to half in an hour but it slightly regained its activity when kept at 4°C. for 24 hours. When the venom solution was heated at 85°C. for 20 minutes part of the venom proteins

were coagulated and the time-concentration curve has a slightly different form indicating that the inhibitor of hæmolysin has been removed (Graph 3). The slight recovery of activity on keeping of the partly heat-inactivated hæmolysin has been

GRAPH 3.
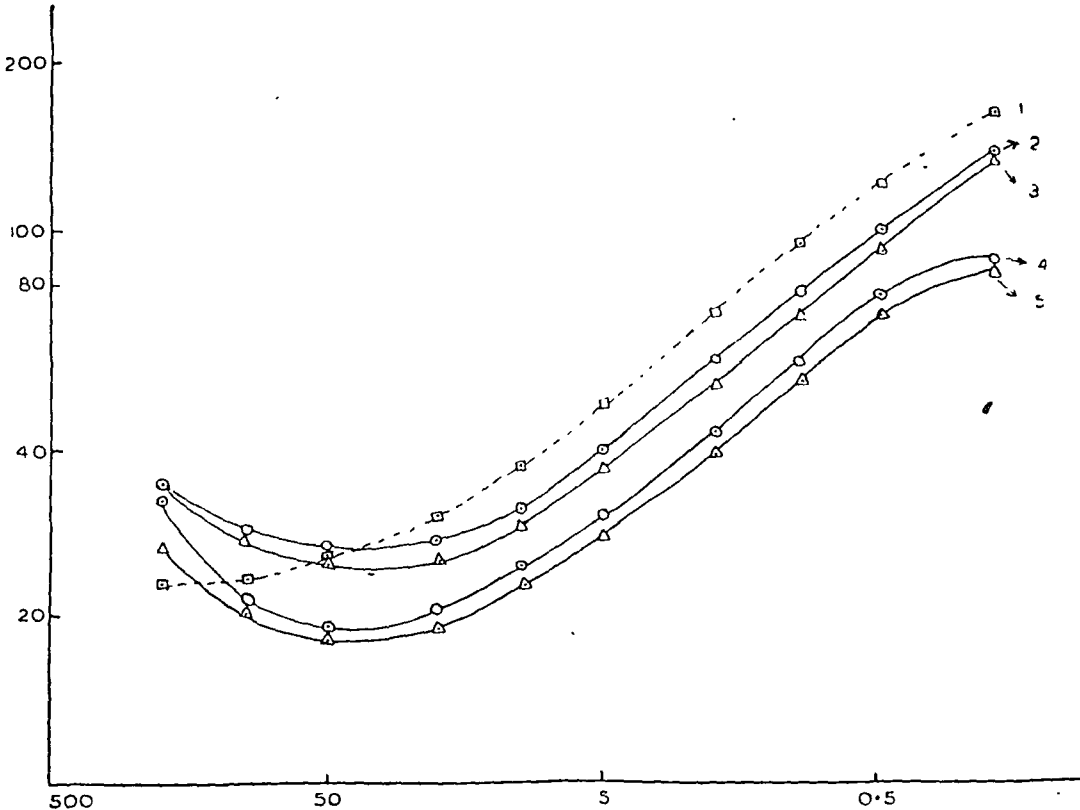
Abscissæ: milligrams of crude cobra venom per c.c. of cells.
Ordinates: time for complete hæmolysis in minutes.
Both are plotted on a logarithmic scale.

GRAPH 3. Effect of heat on hæmolysin in crude venom.

Curve 1. One per cent cobra venom heated at 85°C. for 20 minutes.
  „   2.   „   „   „   75°C. „ one hour.
  „   3. The solution of experiment No. 2 was kept at 4°C. for 24 hours.
  „   4. Control.
  „   5. One per cent cobra venom heated at 60°C. for one hour.

observed by the author in so many cases that there remains no doubt about its reality. The purified hæmolysin is more sensitive to heat than the hæmolysin in crude venom (Graph 4).

GRAPH 4.
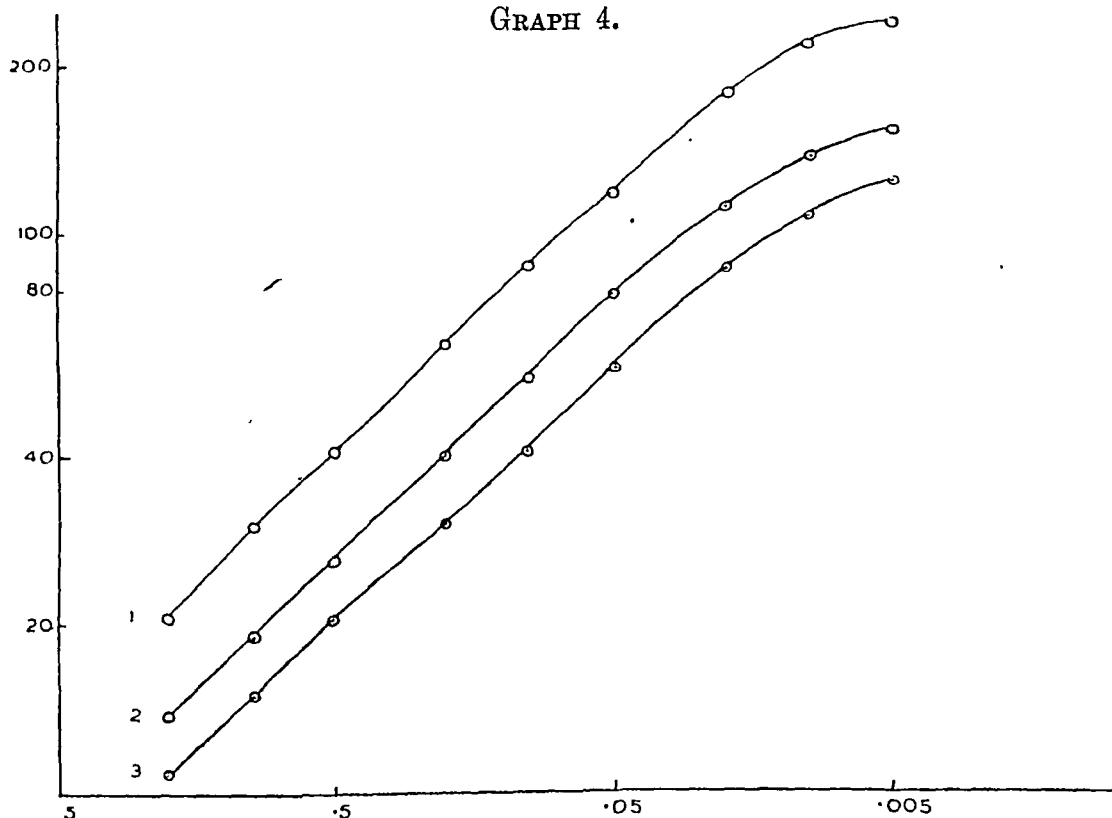
Abscissæ : milligrams of purified hæmolysin per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
        Both are plotted on a logarithmic scale.
GRAPH 4.   Effect of heat on purified hæmolysin.
Curve 1.   0·1 per cent purified hæmolysin heated at 70°C. for 30 minutes.
  „   2.   0·1   „   „   „   „   60°C. „ „   „
  „   3.   Control for Nos. 1 and 2.
The venom solutions were made in acetate, sodium chloride buffer of pH 7·6, and hæmolysis measured in the same buffer solution.

THE EFFECT OF SERUM AND FOREIGN PROTEINS.

Kellaway and Williams (*loc. cit.*) made experiments upon the hæmolysis of horse, human, guinea-pig, and rabbit cells by cobra, death-adder, copper-head, and black tiger snake venoms with and without the addition of inactivated homologous and heterologous sera.   In the case of cobra venom, human and horse sera caused acceleration in the rate of hæmolysis of horse cells, while rabbit and guinea-pig sera caused inhibition.   In the hæmolysis of guinea-pig cells horse serum caused acceleration and the human, rabbit, and guinea-pig sera caused inhibition.

Their results are, however, more or less qualitative and effects due to the variation of the amount of venom and serum have not been studied.

Experimenting with cobra (*Naja Naja*) venom the author noticed that in the hæmolysis of rabbit and guinea-pig cells, the sera of horse, sheep, rabbit, and

guinea-pig, caused inhibition throughout the range of concentration of venom used. In no case acceleration has been observed within this range of concentration of venom.

GRAPH 5.
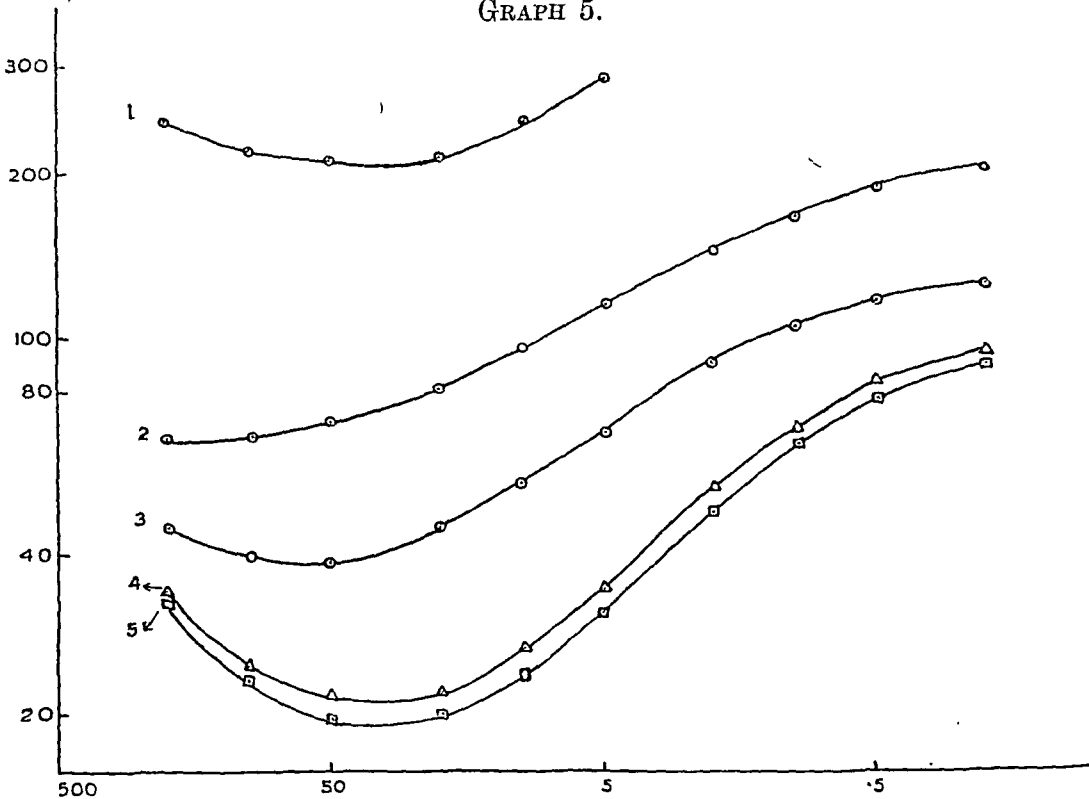
Abscissæ : milligrams of crude cobra venom per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
　　　　　Both are plotted on a logarithmic scale.

GRAPH 5.　Effect of normal serum on hæmolysis by crude cobra venom at pH 7·6.

Curve 1.　2·5 c.c. normal sheep serum per c.c. of red blood cells of guinea-pig.
　„　2.　2·5 c.c. normal rabbit serum per c.c. of red blood cells of guinea-pig.
　„　3.　2·5 c.c. normal guinea-pig serum per c.c. of red blood cells of guinea-pig.
　„　4.　2·5 c.c. normal horse serum per c.c. of red blood cells of guinea-pig.
　„　5.　Crude cobra venom solution without serum.

To obtain the serum used in these experiments, the whole blood was taken in a bottle partially filled with glass-beads and shaken to separate the fibrin. One c.c. of the blood after separation of fibrin was diluted to 10 c.c. with normal saline and then centrifuged. The clear supernatant solution of serum was withdrawn and used in subsequent experiments. Using guinea-pig washed red blood cells, hæmolysis by venom was found to be inhibited by homologous serum. With rabbit, horse, and sheep, serum there was similar inhibition of hæmolysis of the red blood cells of guinea-pig, but sheep serum was found to be a much stronger inhibitor than others (Graph 5). On decreasing the amount of normal serum, no reversal of activity was observed, but the inhibition was decidedly of a lower order. Horse

serum effected no appreciable inhibition. Inhibiting effects of the inactivated sera (heated to 60°C. for some time) of guinea-pig, rabbit, and sheep, were of lower order than those of the corresponding normal sera. On the other hand inactivated horse serum slightly accelerated the hæmolysis of guinea-pig cells. In the case of hæmolysis of rabbit red blood cells similar results were obtained, but the order of inhibition varied.

EFFECT OF LIPOIDS EXTRACTED FROM SERUM ON HÆMOLYSIS.

The total lipoids extracted from the sera of guinea-pig and rabbit have an accelerating effect on the hæmolysis of guinea-pig cells. But lipoids from sheep serum causes inhibition. The total lipoids of the serum was prepared as follows: The original serum was extracted twice with ten volumes of absolute alcohol, care being taken to avoid oxidation. The extract was filtered and evaporated to dryness. The residue was again extracted with absolute alcohol and dried in

GRAPH 6.
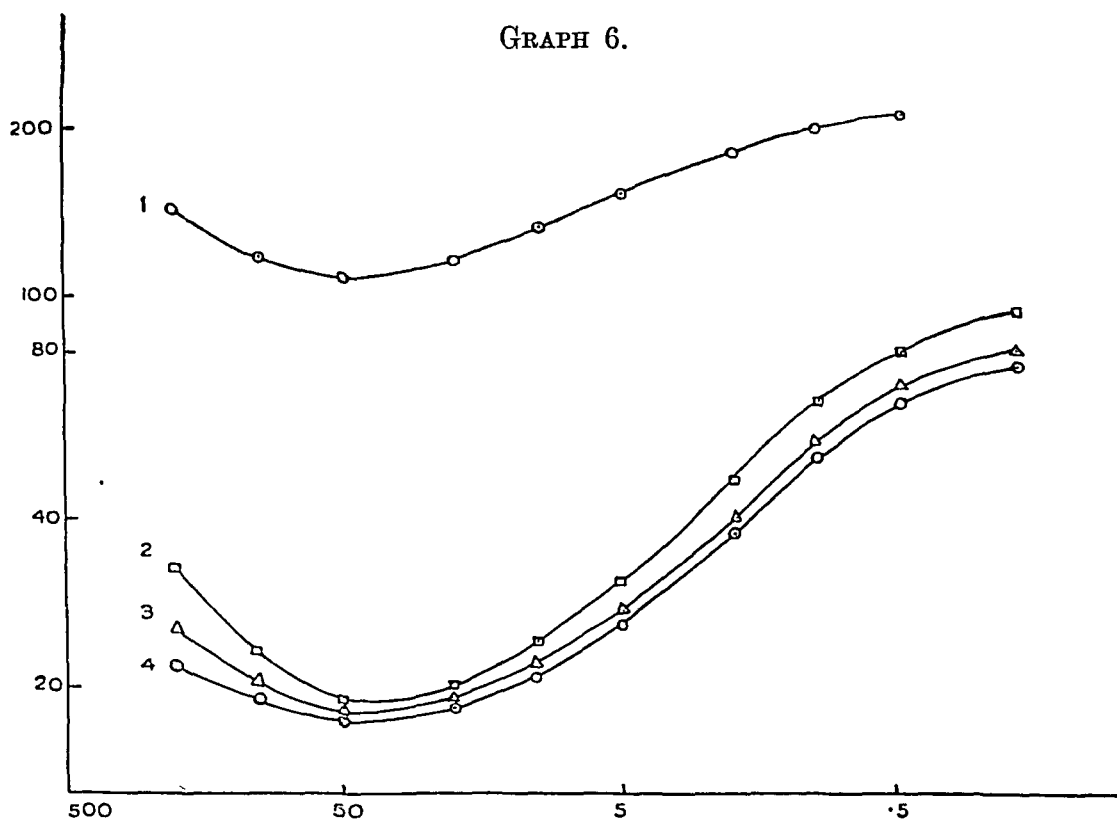
Abscissæ : milligrams of crude cobra venom per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
　　　　　Both are plotted on a logarithmic scale.
GRAPH 6. Effect of lipoids extracted from serum on hæmolysis by crude venom at pH 7·6.
Curve 1. Extract from 2·5 c.c. of sheep serum per c.c. of red blood cells of guinea-pig.
　,,　2. Crude cobra venom solution without serum.
　,,　3. Extract from 2·5 c.c. of rabbit serum per c.c. of red blood cells of guinea-pig.
　,,　4. Extract from 2·5 c.c. of guinea-pig serum per c.c. of red blood cells of guinea-pig.

vacuum. The residue was dissolved in dry ether and the total lipoids precipitated by the addition of two volumes of acetone. The precipitate was then washed with acetone and dried in vacuum and this dried material was used in all subsequent experiments. Extract from 2 c.c. of serum was suspended in 8 c.c. of buffered saline and 0·2 c.c. of it was placed in each tube. The lipoid-free serum acted differently from the lipoids extracted from it (Graph 6). The lipoid-free serum from guinea-pig and rabbit inhibits the hæmolysis of guinea-pig erythrocytes. Lipoid-free sheep serum neither accelerates nor inhibits hæmolysis. It might be suggested that some form of antagonism exists between the proteins and the lipoids in the whole serum, and in majority of cases the effect of proteins far outweighs the effect of the lipoid.

The effect of certain foreign proteins has been studied. Of these 0·2 c.c. of 4 per cent casein caused inhibition of hæmolysis, the same amount of egg-albumin has a faintly accelerating action (Graph 7). The solutions of the proteins were

GRAPH 7.
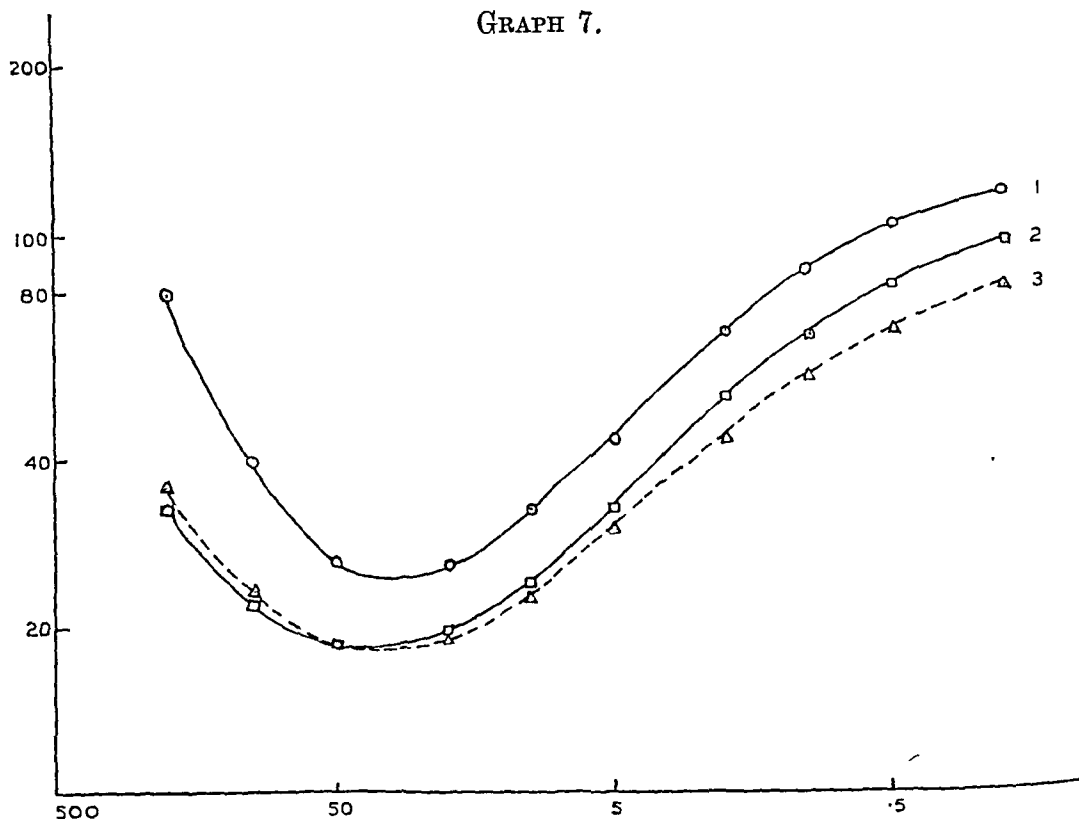
Abscissæ : milligrams of crude cobra venom per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
Both are plotted on a logarithmic scale.

GRAPH 7. Effect of casein and egg-albumin on hæmolysis by crude cobra venom at pH 7·6.

Curve 1. With 0·2 c.c. of 4 per cent casein at pH 7·6.
  ,, 2. Crude cobra venom solution without serum.
  ,, 3. With 0·2 c.c. of 4 per cent egg-albumin at pH 7·6.

adjusted before use to the same concentrations of hydrogen and other ions as used in dissolving the venom and suspending the red blood cells.

EFFECT OF LECITHIN AND CHOLESTEROL.

Kyes and Sachs (*loc. cit.*) showed that hæmolysins act upon the lecithins present in the limiting membrane of the red blood corpuscles. Cells which are not hæmolysed without an activator do not possess in their stroma free lecithin capable of being attached by the enzyme of the venom. They may be hæmolysed by the addition of stroma of susceptible red blood cells. Recently, Ganguly (1937) showed that hæmolysis is also influenced by the chlolesterol content of the cells. The hæmolysis of whole blood is approximately inversely proportional to its cholesterol content and the activation caused by extra-cellular lecithin can be inhibited by the addition of an equivalent amount of cholesterol. Holden (1934) also observed acceleration by hen-ovo-lecithin of hæmolysis of washed rabbit red blood cells by copper-head venom.

GRAPH 8.
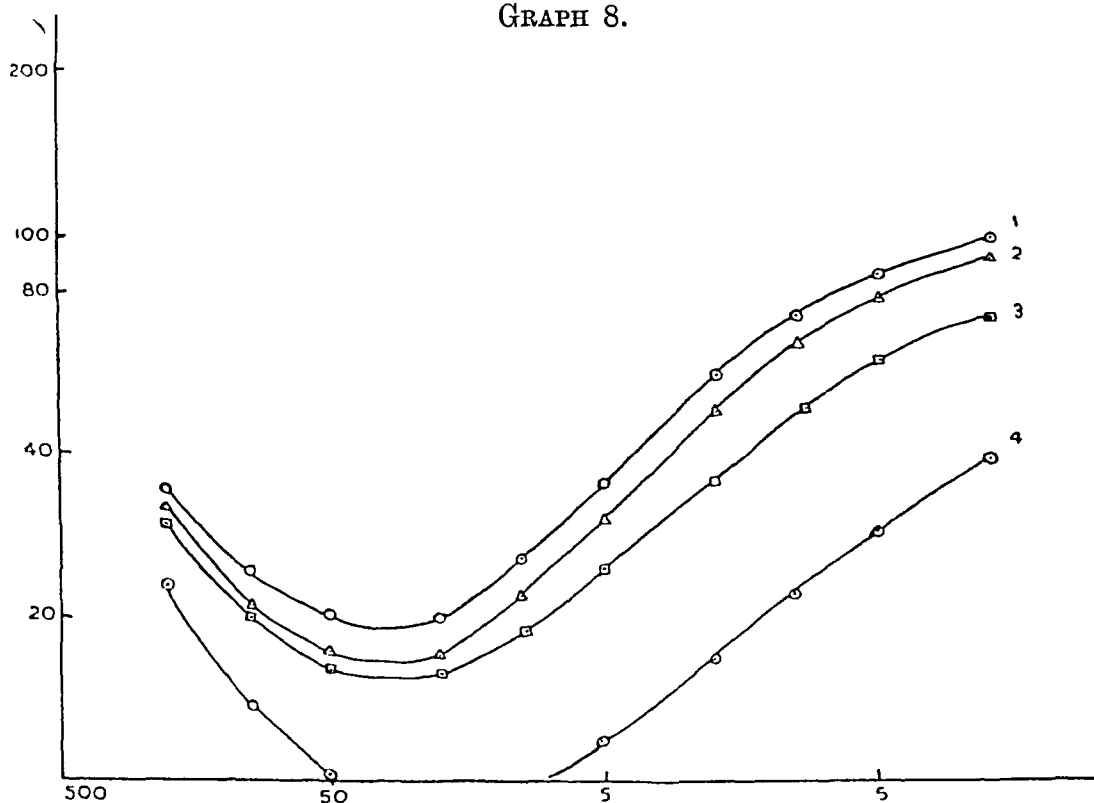
Abscissæ : milligrams of crude cobra venom per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
Both are plotted on a logarithmic scale.

GRAPH 8.   Effect of lecithin and cholesterol at pH 7·6.
Curve 1.   5 mg. cholesterol per c.c. of red blood cells of guinea-pig.
  „   2.   Crude cobra venom solution at pH 7·6.
  „   3.   5 mg. cholesterol and 5 mg. lecithin per c.c. of red blood cells of guinea-pig.
  „   4.   5 mg. lecithin per c.c. of red blood cells of guinea-pig.

The lecithin used in our experiment was Merck's ovo-lecithin purified by repeated solution in ether and precipitation by acetone and the purified product was free from cholesterol. The accelerating effect of lecithin was much influenced by hydrogen-ion concentration. The acceleration at pH 7·6 is more marked than the acceleration at pH 6·0. When cholesterol was mixed with lecithin, the accelerating effect due to the latter was inhibited to a great extent. Cholesterol alone also slightly inhibits unaided hæmolysis of washed red blood cells of guinea-pig (Graph 8). Though ordinarily washed red blood cells of sheep were not hæmolysed by cobra venom, it could be hæmolysed by the addition of lecithin.

EFFECT OF SALTS AND OTHER NON-SPECIFIC SUBSTANCES.

The effect of $CaCl_2$ on copper-head hæmolysin has been studied by Holden (1935). He found that variation in the concentration of the salt caused either
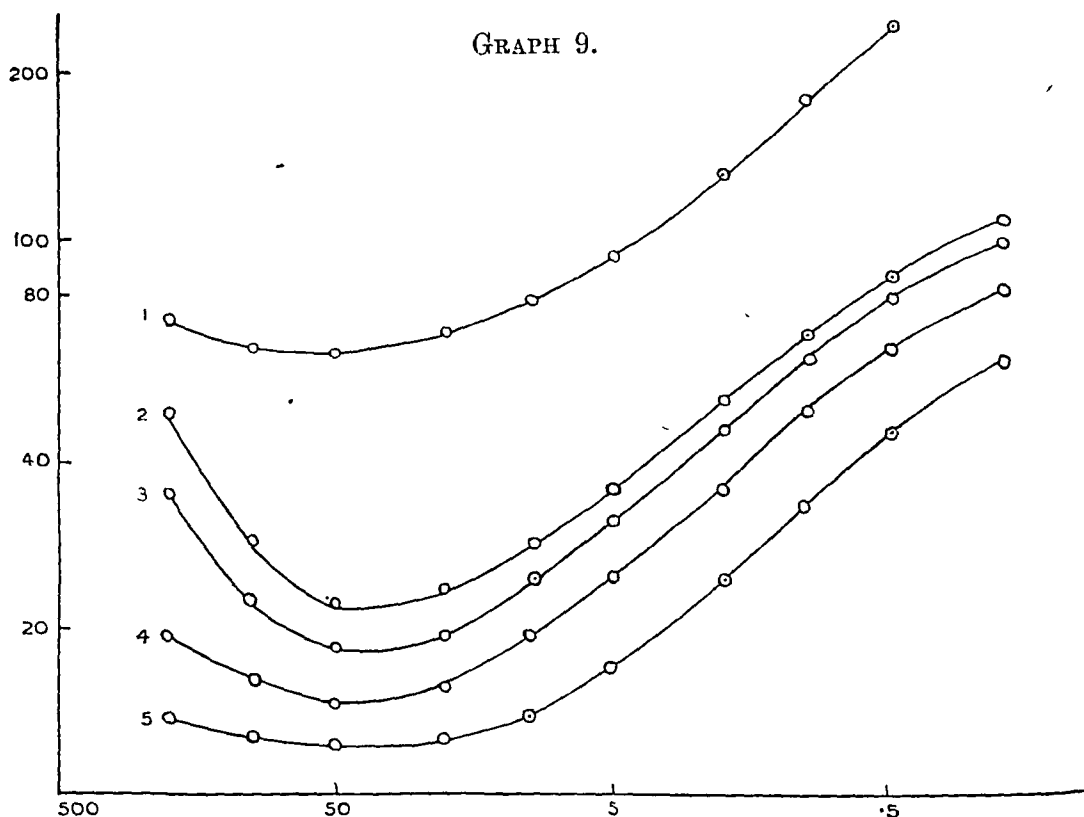
GRAPH 9.

Abscissæ : milligrams of crude cobra venom per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
            Both are plotted on a logarithmic scale.
GRAPH 9. Effect of salts on hæmolysis by crude venom at pH 7·6.
Curve 1. 5 mg. $PbCl_2$ per c.c. of red blood cells of guinea-pig.
  „   2. 5 mg. $BaCl_2$ „   „   „   „   „
  „   3. Crude cobra venom solution at pH 7·6.
  „   4. 5 mg. $HgCl_2$ per c.c. of red blood cells of guinea-pig.
  „   5. 5 mg. $CaCl_2$ „   „   „   „   „

acceleration or inhibition. Similar effects have been observed with cobra venom using red blood cells of guinea-pig. Five mg. of anhydrous calcium chloride per c.c. of red blood cells caused acceleration of hæmolysis but with 25 mg. of the salt marked inhibition was observed.

Barium chloride and lead chloride caused inhibition of hæmolysis. The behaviour of $HgCl_2$ is rather peculiar. Hæmolytic activity is found to be increased when measured soon after the addition of $HgCl_2$ but when $HgCl_2$ is allowed to act on the venom for two hours inhibition occurred. With purified hæmolysin there was always inhibition of hæmolytic activity. Of some non-specific substances tried glycine accelerated hæmolysis (Graphs 9 and 10).

GRAPH 10.
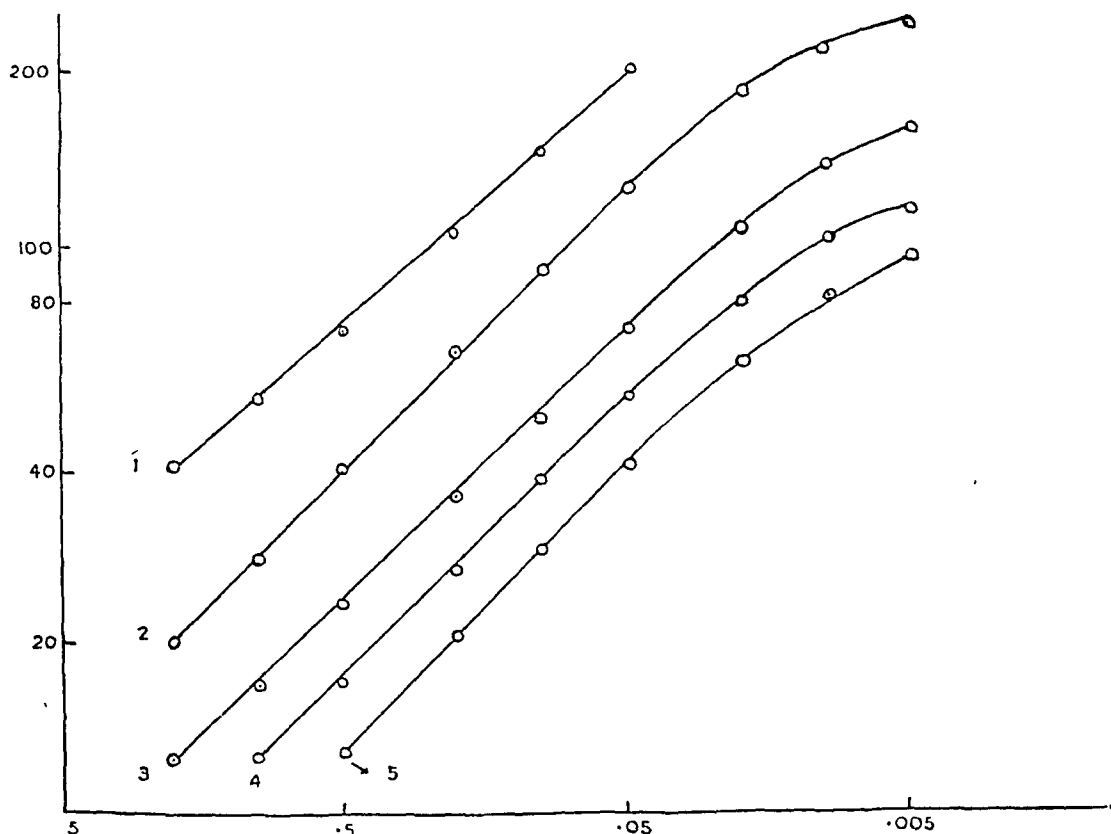
Abscissæ : milligrams of crude cobra venom per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
Both are plotted on a logarithmic scale.

GRAPH 10. Effect of salts and glycine on hæmolysis by purified hæmolysin at pH 7·6.

Curve 1. 5 mg. $PbCl_2$ per c.c. of red blood cells of guinea-pig.
  „ 2. 5 mg. $BaCl_2$ „ „ „ „ „
  „ 3. Purified hæmolysin at pH 7·6.
  „ 4. 20 mg. glycine per c.c. of red blood cells of guinea-pig.
  „ 5. 5 mg. $CaCl_2$ „ „ „ „ „

The experiments do not elucidate the mechanism but rather the complex nature of hæmolytic action is emphasized. According to Herrmann and Rohner (*loc. cit.*) it can be suggested that all these substances cause changes in the colloidal system composed of protein, lecithin, and cholesterol, which constitute the limiting surface of the red blood corpuscles.

SUMMARY.

1. The hæmolytic activity of cobra hæmolysin is maximum at pH 7·6.

2. When cobra venom solution is heated at 60°C. for an hour its hæmolytic activity is not diminished but slightly increased; heating at higher temperature, however, causes loss in hæmolytic activity.

3. Normal sera of the horse, sheep, rabbit, and guinea-pig, inhibit the hæmolysis of the red blood cells of rabbit and guinea-pig by cobra venom, inactivated serum behaves similarly but the inhibition was of lower magnitude than the normal serum. Inactivated horse serum slightly accelerates hæmolysis.

4. The lipoids extracted from the sera of guinea-pig and rabbit accelerate the hæmolysis of red blood cells of guinea-pig but lipoids from sheep serum cause inhibition.

5. Casein causes inhibition of hæmolysis of red blood cells of guinea-pig, but egg-albumin slightly accelerates its hæmolysis.

6. Lecithin accelerates the hæmolysis of red blood cells of guinea-pig by cobra venom and the effect is more marked at pH 7·6 than at pH 6·0, while cholesterol inhibits hæmolysis.

7. Higher concentration of $CaCl_2$ inhibits hæmolysis of red blood cells of guinea-pig but lower concentration accelerates hæmolysis. $PbCl_2$ and $BaCl_2$ inhibit hæmolysis. $HgCl_2$ on long standing in contact with cobra venom inhibits its hæmolytic activity. Hæmolytic activity of purified hæmolysin is increased with the addition of glycine.

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STUDIES ON HÆMOLYSIN OF COBRA VENOM.

Part III.

REVERSIBLE INACTIVATION OF HÆMOLYSIN OF COBRA (*NAJA NAJA*) VENOM.

BY

S. S. DE, M.SC.

(*Inquiry under the Indian Research Fund Association, University College of Science & Technology, Calcutta.*)

OF the various chemical and physical factors controlling the activity of an enzyme, the necessity for adequate control of hydrogen-ion activity in enzymic studies has been demonstrated by Sφrensen (1907) in a series of papers. In determining the activity of an enzyme the activity pH curves should be evaluated first. The activity of an enzyme may also be altered through various 'salt effects'. The presence of added ions or molecules would cause 'salt effect' through a change in the thermodynamic environment in the reaction mixture. The activation and inactivation caused by various substances in enzymic actions have been discussed in some detail by Haldane in his book 'Enzymes'. The activating power of these reagents has been interpreted in terms of salt effects, co-enzyme actions, changes in the oxidation reduction state of their specific activator, removal of inhibiting metal ions, etc.

Inhibiting effect of heavy metals salts like $AgNO_3$, $HgCl_2$, upon saccharase is known from early times. This inhibition can be reversed by treatment with H_2S (Euler and Svanberg, 1920). Mendel and Blood (1910) observed that the rate of proteolysis by papain was enhanced by compounds like HCN and H_2S. Grassmann *et al.* (1929) showed that papain and kathepsin can be activated by organic sulphydryl compounds such as glutathione and cysteine. Similar activation has been observed with the enzyme urease by Hellermann *et al.* (1933). Perlzweig (1932) suggested that the association of urease with a sulphydryl activator can account for the re-activation by cyanide, hydrogen sulphide, and by other reagents of urease preparations inactivated by the ions of the heavy metals. The inactivation would consist of the metal-ion catalysing the oxidation of the activator; regeneration of which might result from the reducing action of the cyanides or sulphides. But Summer and Poland (1933) showed that sulphydryl compounds

may be present in jack-bean meal. Crystallized and dialysed enzymes possess characteristic activity even in the absence of supplementary thiol groups.

Perlzweig (*loc. cit.*) observed that crystalline urease was partially inactivated through æration and simultaneously a weakening of the nitroprusside test for thiol was observed. Hellermann and Perkins (1934) proposed that the phenomenon of inactivation and re-activation is due to oxidation and reduction of the substituent sulphydryl groups, of the enzyme itself and not to changes with the associated activator. The enzyme would then be active in its reduced form, e.g., En-S-H and reversibly inactivated by controlled oxidation to an inactive form En-S-S-En.

According to Bersin (1936) ' The active enzyme is thus to be looked upon as a thiol compound while its inactive form is a disulphide compound '.

This may be represented as follows :—

$$2 \text{ En-SH} \xrightarrow[\text{Reduction}]{\text{Oxidation}} \text{En-S-S-En} + 2\,(H) \qquad .. \quad (1)$$

(Active)   (Inactive)
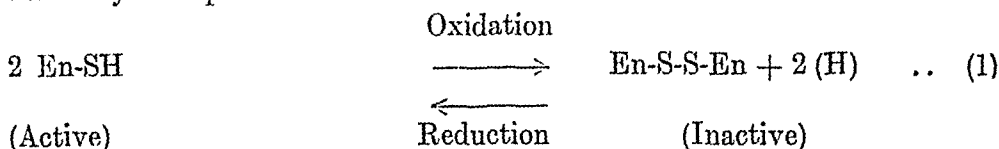
The reduction of the dithio form by cysteine or glutathione (R-SH) may be represented in the form thus—

$$\text{En-S-S-En} + 2\,R - SH \rightarrow 2\,\text{En-SH} + \text{R-S-S-R} \qquad .. \qquad .. \quad (2)$$
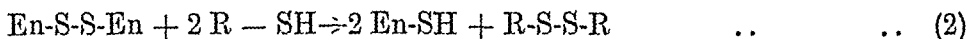
Hydrogen cyanide or hydrogen sulphide react with the dithio form of the enzyme in the following ways :—

$$\text{En-S-S-En} + HCN \rightarrow \text{En-SH} + \text{En-SCN} \qquad .. \qquad .. \qquad .. \quad (3)$$
$$\text{En-S-S-En} + HSH \rightarrow \text{En-SH} + \text{En-S-SH} \qquad .. \qquad .. \qquad .. \quad (4)$$
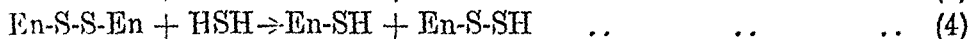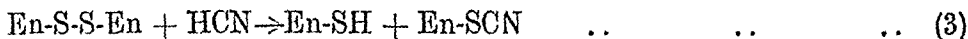
It has been shown in the case of urease and papain that cysteine activates the inactive preparations of the enzyme to a much higher level than hydrogen cyanide or hydrogen sulphide. Such differences are very probably not due only to relative rates of reaction but to actual variations in the mode of chemical attack. From equations (2) to (4) it is evident that by the action of cysteine more of the active material will be produced from the same amount of oxidized enzyme than with hydrogen cyanide or hydrogen sulphide. Secondary actions may produce more of the thiol form by the hydrolysis of the product En-SCN.

It has also been observed that the enzyme urease can be inactivated by treatment with cuprous oxide or certain organic mercurials (e.g., phenylmercuric chloride). This inhibiting action was also found to be reversible.

The formation of the mercaptides has been represented by the following type of equations :—

$$2\text{En-SH} + Cu_2O \rightarrow 2\text{En-S-Cu} + H_2O$$
$$\text{En-SH} + C_6H_5HgCl \rightarrow \text{En-S} + Hg\text{-}C_6H_5 + HCl.$$
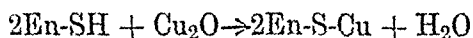
Working with pneumococcal hæmolysin Avery and Neill (1924) inactivated it by treatment with air and H_2O_2. Subsequently, Neill (1926) restored the activity of the inactive product by treatment with $Na_2S_2O_4$ or with certain anærobic bacteria.

Shwachman *et al.* (1934) inactivated hæmolysin extract from frozen and thawed pneumococci by suitable oxidizing agents and mercaptide-forming compounds such

as Cu_2O and certain organo-mercurials. The hæmolysin inactivated by oxidizing agents was re-activated by many thiol compounds and ·by other reducing agents, e.g., cyanide, ascorbic acid plus iodine, and hæmolysin inactivated by metallic compounds was regenerated by H_2S and cysteine.

Hopkins and Morgan (1938) showed that when the enzyme succinic dehydrogenase is exposed to the oxidizing influence of GSSG its activity disappears simultaneously with the disappearance of the tissue thiol groups, while both reappear together under the reducing influence of GSH (glutathione reduced). The activity of the enzymes, lipases, phosphatases, cerebrosidase, and glucosidase, is related in one way or another to sulphur grouping has also been shown. Assuming that hæmolysin of cobra venom may be similar to pneumococcal hæmolysin the effect of oxidizing agents like iodine, hydrogen peroxide, ferricyanide, etc., on its activity was studied by the author. It was found that the inactivation caused by controlled oxidation with reagents mentioned above was reversed by hydrogen sulphide, organic sulphydryl compounds and other reducing agents. The inactivation by metallic derivatives and the reversals thereof has also been observed. Both the crude venom and the purified hæmolysin (De, 1939) were subjected to the action of the above reagents, and some peculiarities in their behaviour have been observed. Using crude venom the hæmolytic activity was increased with low amounts of the oxidizing agents, but moderately high amounts of them acting for a long period ultimately reduced the hæmolytic activity. The effect of oxidation on purified hæmolysin always resulted in inactivation of the product.

PROCEDURE.

Twenty-five mg. of crude cobra venom were used for each experiment. The venom was dissolved in 4 c.c. of buffered saline of pH 7·6 and various amounts of the oxidizing agents adjusted to the desired hydrogen-ion concentration were added in volumes of 1 c.c. and allowed to react for definite periods. Two c.c. of the mixture were then pipetted out and diluted to 10 c.c., and after further adequate dilution the hæmolytic activity of this fraction was compared with the control venom solution which has been maintained side by side. Simultaneously, 2 c.c. fraction from the remaining mixture were subjected to the action of reducing agents. If acidic reagents were added they were neutralized with Na_2CO_3 before comparing the lytic activity. With purified hæmolysin the usual procedure was the same but only 5 mg. of the purified sample was used. The hæmolytic activity was determined as mentioned in Part II of this paper (page 793, this issue). To graded amounts of venom made up to 1·0 c.c. was added 0·4 c.c. of washed 5 per cent suspension of red blood cells of guinea-pig and as usual the reaction of the medium was maintained at pH 7·6. It will be noticed that though the control curve varies slightly with time, the variation is insignificant in comparison with the curves obtained with inactivated and the re-activated product. The purified hæmolysin gave faint test for sulphydryl sulphur with sodium nitroprusside. After reduction with NaCN however a stronger test was obtained. It may be maintained in this connection that ergothionine and thiosalicylate ion do not respond to this test. Sulphydryl groups if present in the molecule of hæmolysin in the two forms mentioned are probably not detected by this reagent.

INACTIVATION BY IODINE.

The iodine solution used was $0·05N$ in $0·05M$ Kl. Four c.c. of venom solutions containing 25 mg. of crude cobra venom were placed in four flasks and to them were added respectively (1) 0·1 c.c , (2) 0·2 c.c., (3) 0·5 c.c., and (4) 0·8 c.c., of the above iodine solution, and the total volume made up to 5 c.c. with saline in each flask. After one hour 1 c.c. fraction from each was taken out and the hæmolytic activity compared with the control. In (1), (2), and (3), there was an increase of hæmolytic activity, but in (4) the activity was much depressed (Graph 1). After two hours' standing only (3) showed the depression of activity and after 3 hours' incubation no lowering of activity was observed in (1) and (2).

GRAPH 1.
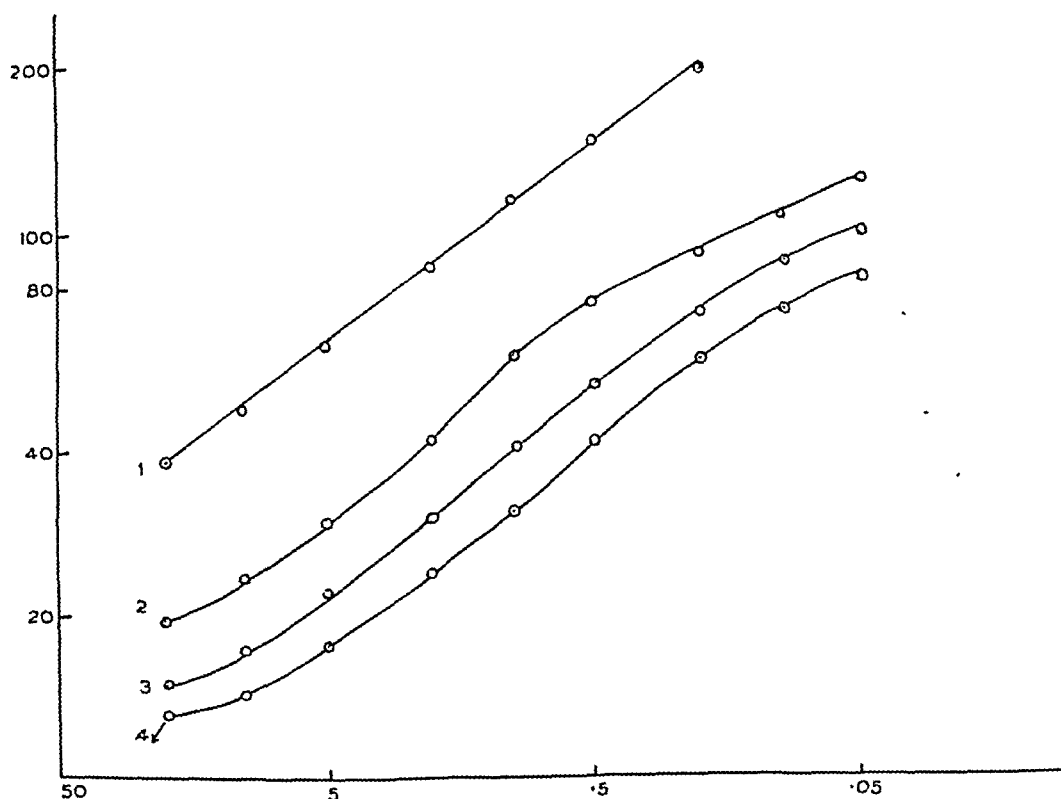
Abscissæ : milligrams of crude venom per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
Both are plotted on a logarithmic scale.

GRAPH 1. Effect of varying amounts of iodine on the activity of hæmolysin in crude venom.

Curve 1. To 25 mg. of crude venom was added 0·8 c.c. of 0·05N iodine solution.
  ,,   2. Crude cobra venom solution at pH 7·6.
  ,,   3. To 25 mg. of crude venom was added 0·5 c.c. of 0·05N iodine solution.
  ,,   4.   ,,   ,,   ,,   ,,   0·2 c.c.   ,,   ,,   ,,

The iodine solution was allowed to act for one hour and hæmolysis then measured with acetate buffer of pH 7·6.

The experiments were then repeated with larger amounts of iodine as indicated in (4), and allowed to stand for an hour. Ten mg. portions of the partly inactivated product were treated with H_2S, NaCN, ascorbic acid, cysteine, and reduced glutathione. These reagents brought about partial or complete re-activation; H_2S, cysteine and reduced glutathione brought back the full activity, whereas ascorbic acid and sodium cyanide only partially re-activated the product. But when the iodine was allowed to react for longer periods complete reversal of activity was not
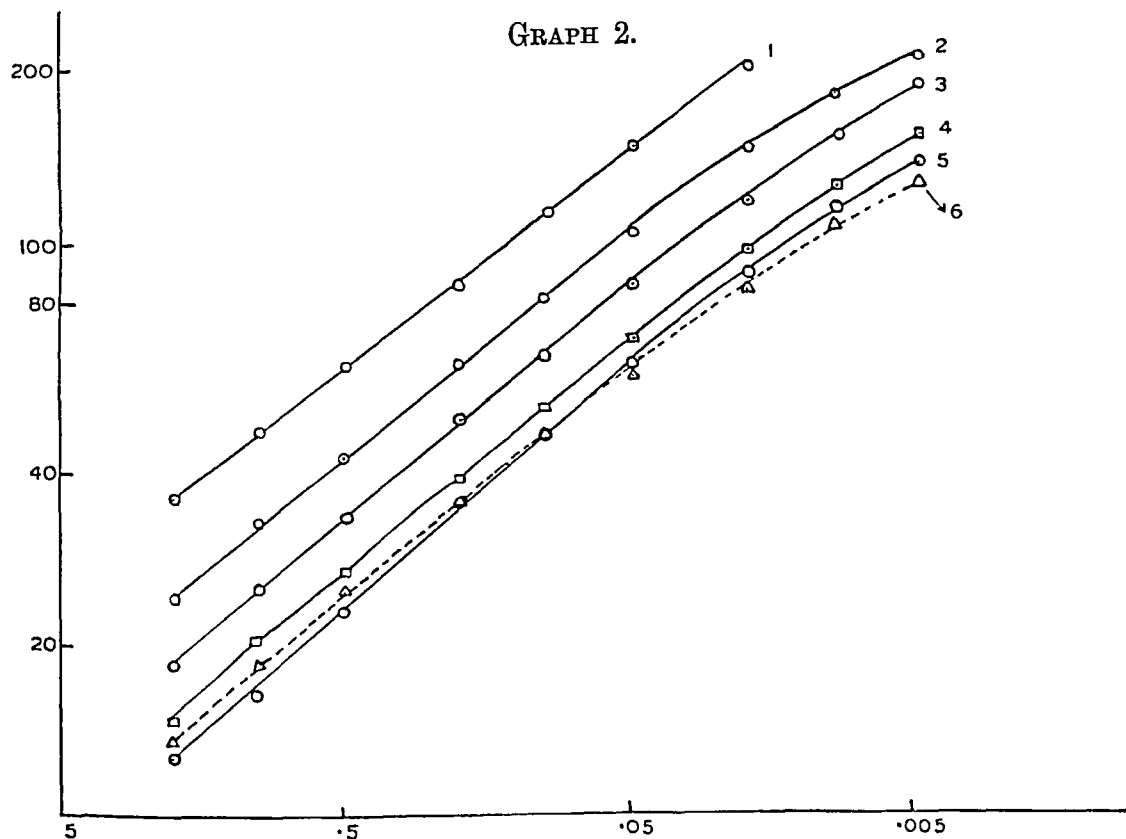
GRAPH 2.

Abscissæ : milligrams of purified hæmolysin per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
Both are plotted on a logarithmic scale.

GRAPH 2. Inactivation of purified hæmolysin and its re-activation by various reducing substances.

Curve 1. To 25 mg. of purified hæmolysin was added 0·5 c.c. of 0·05N iodine solution and allowed to act for 30 minutes.
,, 2. 5 mg. fraction from No. 1 + 0·3 c.c. of 0·1M ascorbic acid.
,, 3. ,, ,, ,, + 0·3 c.c. of 0·1M NaCN.
,, 4. ,, ,, ,, + 0·3 c.c. of 0·1M cysteine.
,, 5. Purified hæmolysin solution at pH 7·6.
,, 6. 5 mg. fraction from No. 1 + 0·3 c.c. of 0·07M H_2S.

The reductants were allowed to act for 90 minutes and hæmolysis measured with the above-mentioned buffer solution.

noticed.   The action of iodine on purified hæmolysin always produced inactivation of the product.   The action was also more rapid and drastic with comparable amounts of iodine.   To 5 mg. of purified hæmolysin was added 0·1 c.c. of the iodine solution, and the degree of inactivation after 30 minutes was more marked than in the case of crude venom.   The effect of the reducing agents were the same as in the case of crude venom (Graph 2).   The activators were generally allowed to act for 90 minutes.   The sensitivity of purified hæmolysin to oxidation is far greater than that of crude venom.

Inactivation by hydrogen peroxide.—The effect of hydrogen peroxide on crude venom is similar to that of iodine.   The inactivation of purified hæmolysin by comparable amounts of hydrogen peroxide as was used in the case of iodine, and its re-activation with cysteine and glutathione is illustrated in Graph 3.   Hydrogen peroxide was allowed to act for 30 minutes and the activators for 90 minutes.
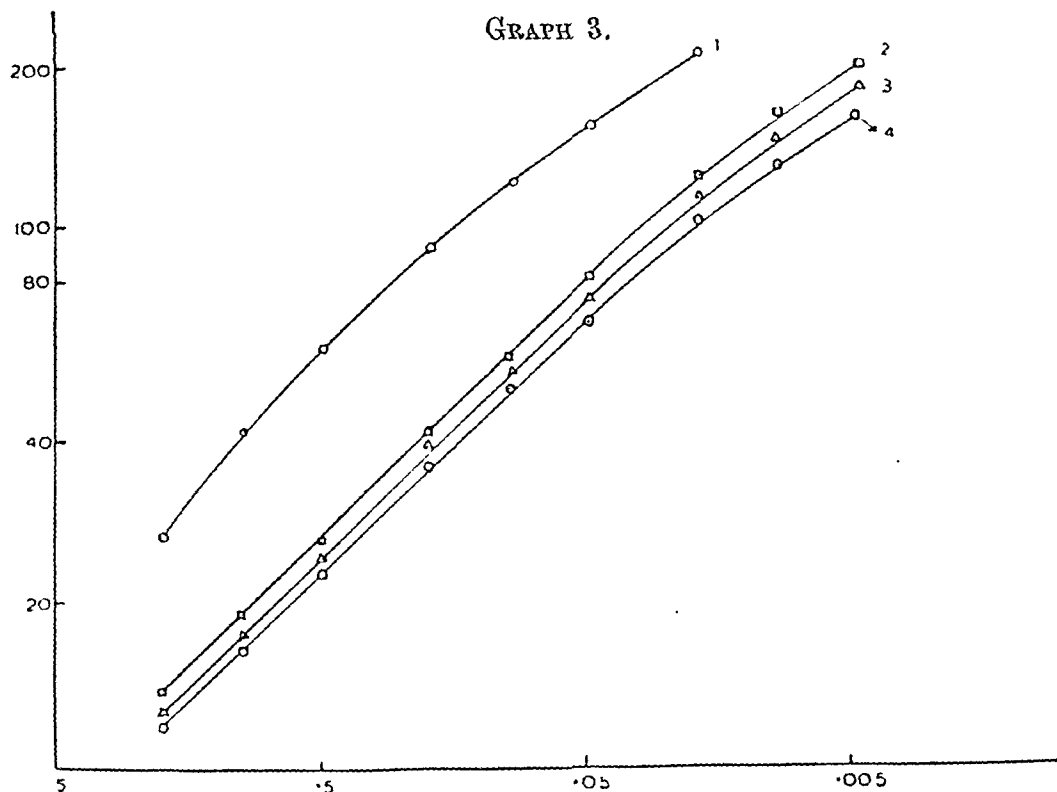
GRAPH 3.

Abscissæ: milligrams of purified hæmolysin per c.c. of cells.
Ordinates: time for complete hæmolysis in minutes.
           Both are plotted on a logarithmic scale.

GRAPH 3.   Inactivation of purified hæmolysin by hydrogen peroxide and its re-activation.

Curve 1.   To 20 mg. of purified hæmolysin was added 0·4 c.c. of 0·05N H_2O_2 solution and
              allowed to act for 30 minutes.
    „   2.   5 mg. fraction from No. 1 + 0·3 c.c. of 0·1M cysteine (pH 4·2).
    „   3.   „   „   „   + 0·3 c.c. of 0·1M reduced glutathione (pH 4·8).
    „   4.   Control.

Inactivation by ferricyanide.—The action of ferricyanide on crude venom was not so pronounced as with iodine or hydrogen peroxide. The activation and inactivation of hæmolysin in crude venom was less than that produced by comparable concentrations of iodine or hydrogen peroxide. As expected the effect was also less on purified hæmolysin (Graph 4). In this connection parallel

GRAPH 4.
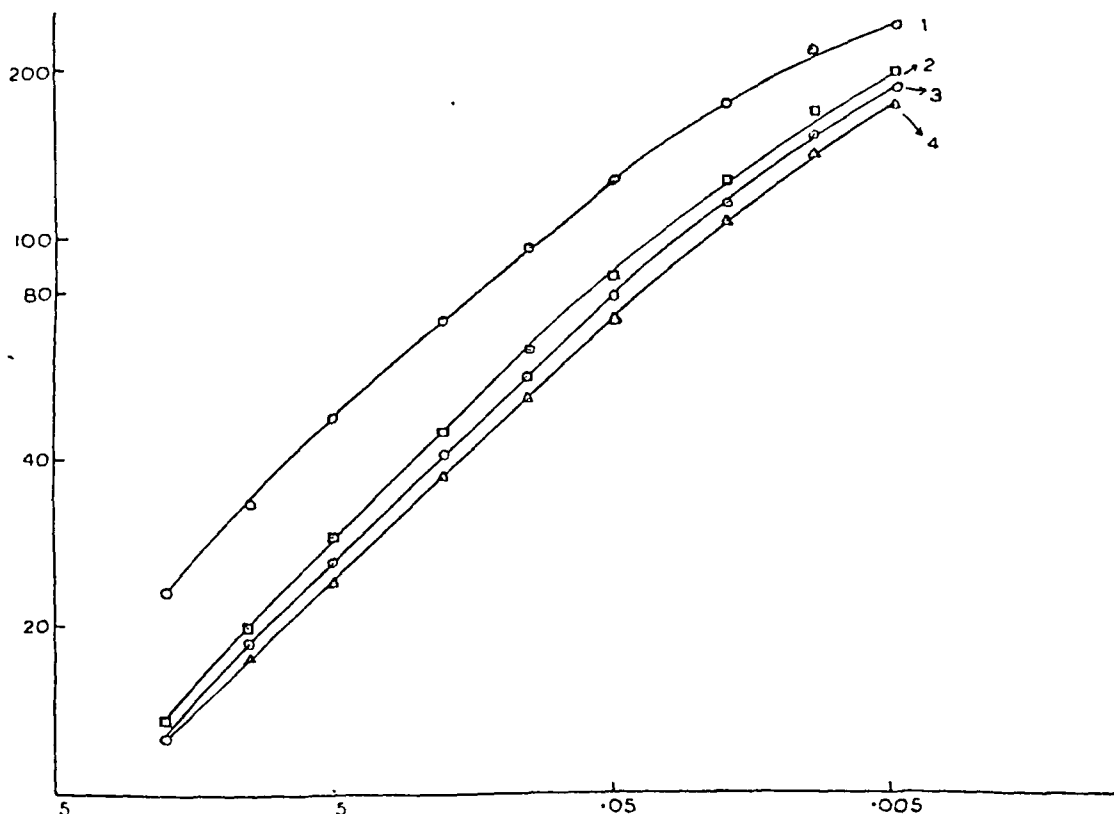
Abscissæ : milligrams of purified hæmolysin per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
Both are plotted on a logarithmic scale.

GRAPH 4. Inactivation of purified hæmolysin by ferricyanide and its re-activation.

Curve 1. To 20 mg. of purified hæmolysin was added 0·4 c.c. of 0·05N $K_3F(CN)_6$ solution and allowed to act for 30 minutes.
„ 2. 5 mg. fraction from No. 1 + 0·3 c.c. of 0·1M cysteine (pH 4·2).
„ 3. „   „   „   + 0·3 c.c. of 0·07M H_2S.
„ 4. Purified hæmolysin solution at pH 7·6.

experiments were undertaken with ferricyanide, but no depression in the hæmolytic activity of the purified sample was observed.

Action of benzoquinone.—Benzoquinone had practically no action on hæmolysin in crude venom, but its effect on the purified product is drastic and a noticeable inactivation was observed in 10 minutes. Its activity was restored partially with

H$_2$S only (Graph 5). Under all the conditions studied the inactivation caused by quinone could not be fully reversed. The fact suggests that quinone has a specific reactivity to hæmolysin in addition to its oxidizing capacity.

GRAPH 5.
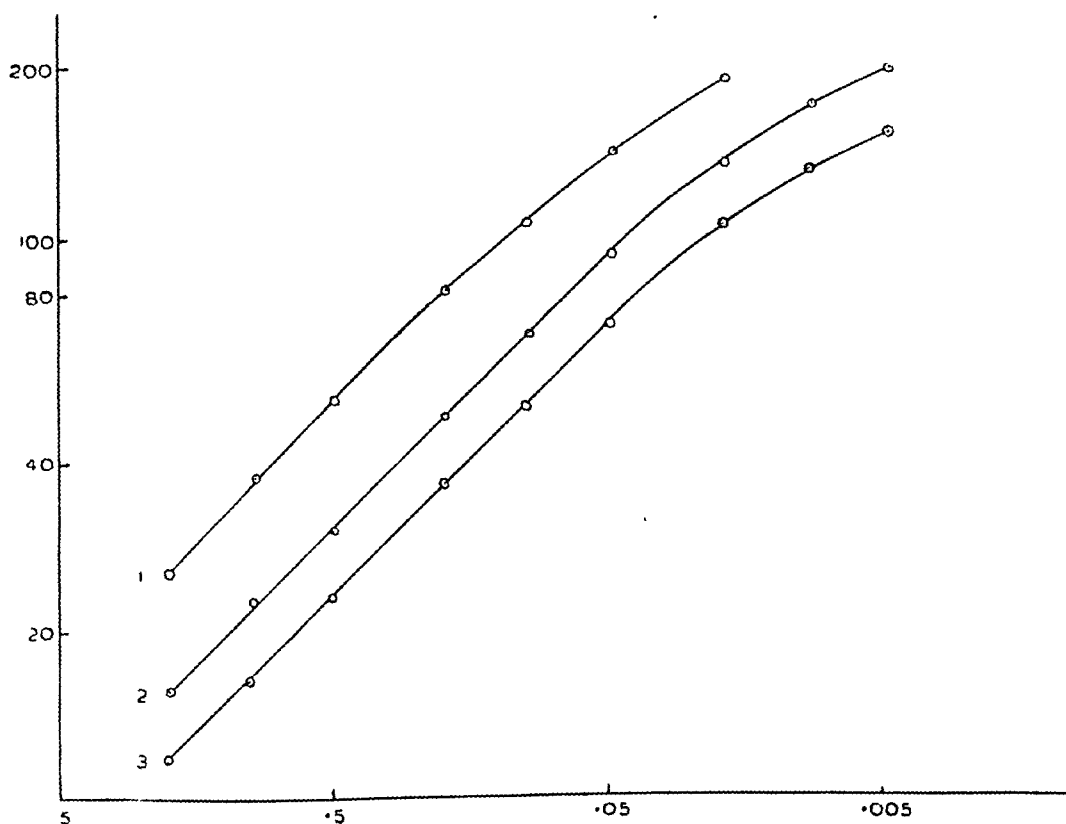
Abscissæ : milligrams of purified hæmolysin per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
            Both are plotted on a logarithmic scale.

GRAPH 5.   Inactivation of purified hæmolysin by benzoquinone and its re-activation by hydrogen sulphide.

Curve 1.   To 10 mg. purified hæmolysin was added 1·1 mg. benzoquinone and allowed to react for 10 minutes.

   ,,   2.   5 mg. fraction from No. 1 + 0·5 c.c. of 0·07M H$_2$S.

   ,,   3.   Purified hæmolysin solution at pH 7·6.

Action of cuprous oxide and mercuri-organic derivatives.—It is known that silver and mercuric salts, cuprous oxide and aryl or alkyl mercuric salts or hydroxides, form mercaptides with sulphydryl compounds. Hopkins (1929) isolated glutathione by forming the Cu salt of glutathione. Shwachman *et al.* (*loc. cit.*) observed that cuprous oxide produced inactivation of pneumococcal lysin which is partly reversible. Organo-mercurials of the type R-Hg-X (where X = Cl or OH) also produced reversible inactivation. The author also found that cuprous oxide acting

on crude venom activated the hæmolysin during the earlier periods but on keeping the ultimate result was the inactivation of the product. To 25 mg. of the purified product were added 6 mg. of freshly-prepared cuprous oxide and the whole allowed to stand for one hour. The excess cuprous oxide was centrifuged out. The inactivation and re-activation is indicated by the curves in Graph 6. Inactivation

GRAPH 6.
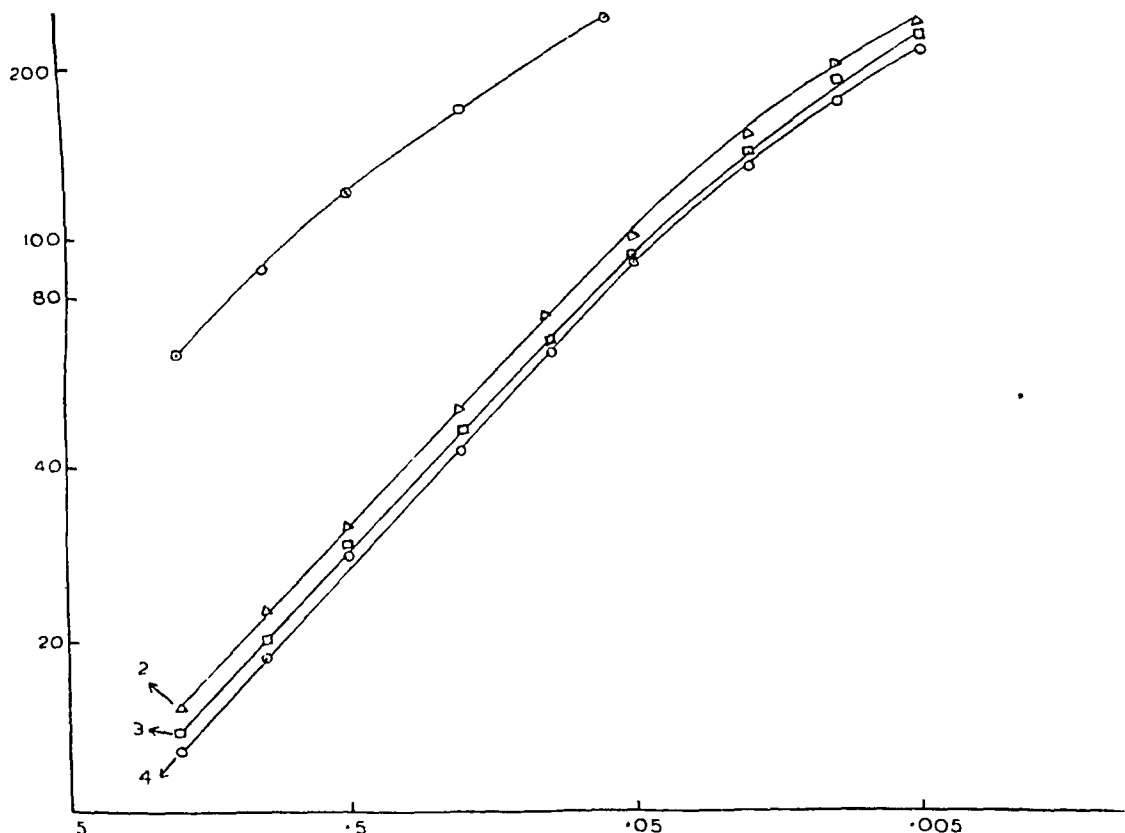
Abscissæ : milligrams of purified hæmolysin per c.c. of cells.
Ordinates : time for complete hæmolysis in minutes.
    Both are plotted on a logarithmic scale.

GRAPH 6.   Inactivation of purified hæmolysin and its re-activation by reduced glutathione and hydrogen sulphide.

Curve 1.   To 25 mg. purified hæmolysin was added 5 mg. Cu$_2$O and allowed to stand for one hour and then the mixtures centrifuged to remove the excess Cu$_2$O.
   „   2.   5 mg. fraction from No. 1 + 0·3 c.c. of 0·1M reduced glutathione (pH 4·8).
   „   3.   „   „   „   + 0·3 c.c. of 0·07M H$_2$S.
   „   4.   Purified hæmolysin solution at pH 7·6.

by C$_6$H$_5$ Hg OH or C$_6$H$_5$ HgCl has been restored by subsequent treatment with H$_2$S and glutathione. Of particular interest in this connection is the observation that bis-p-tolyl mercury which cannot form mercaptide produced no measurable inactivation of hæmolysin.

DISCUSSION.

The experiments with crude venom described above show unequivocally that the oxidizing agents tried, preferentially act on the inhibitor of hæmolysin rendering it ineffective as inhibitor. The inhibitor is probably adsorbed on the surface of hæmolysin molecules and it being oxidized first protects the hæmolysin molecules from oxidation. The sensitivity of purified hæmolysin towards oxidation also suggests that in crude venom there is a substance which protects hæmolysin molecules from oxidation. Of all the oxidizing agents tried the inactivation caused by benzoquinone cannot be fully reversed. Under all conditions studied with benzoquinone only a partial re-activation has been noticed. This irreversible action of quinone can be attributed to an attack through the reactive groups of the quinone in addition to direct oxidation. The behaviour of cobra hæmolysin towards reversible oxidation by the oxidizing agents is similar to that which has been observed with pneumococcal hæmolysin. The parallelism in behaviour of hæmolysin towards oxidizing and reducing substances and towards mercaptide-forming substances on the one hand and of many other enzymes (urease, papain, and kathepsin) toward those reagents is of outstanding importance. The following observations all lead to testify that sulphydryl groups control the activity of hæmolysin :—

    (*a*) Cobra hæmolysin is inactivated by ferricyanide at a rate much slower than that produced by comparable concentrations of iodine. The above oxidants behave similarly with thiol compounds.

    (*b*) Cobra hæmolysin is reversibly inactivated by Cu_2O which is specific for thiol compounds forming mercaptides.

    (*c*) Mercaptide-forming organo-mercurials also inactivates the hæmolysin reversibly.

    (*d*) The organo-mercurials of the type R-Hg-X which cannot form a mercaptide with thiol compounds do not inactivate hæmolysin.

Irreversible inactivations with iodine were readily obtained with larger amounts of iodine or prolonged action of the oxidant. It is probable that oxidation of sulphur atoms to a stage beyond the dithio configuration would cause irreversible action.

SUMMARY.

1. The hæmolytic activity of crude cobra-venom solution is first increased but finally depressed on standing in contact with a moderate concentration of iodine solution but with lower concentration the activity increases and no reversal of activity is obtained. The partly inactivated product regains its activity on treatment with H_2S, NaCN, ascorbic acid, cysteine, and reduced glutathione. With purified hæmolysin these resulted always in inactivation of the product and its activity was regained with the reagents tried with crude venom.

2. Behaviour of hydrogen peroxide and ferricyanide is similar to that of iodine but the effect of ferricyanide is less pronounced than that of iodine.

3. Effect of benzoquinone is rather peculiar, it has no action on crude venom, but depresses the activity of purified hæmolysin in 10 minutes which is partially regenerated by hydrogen sulphide.

4. Cuprous oxide and phenyl-mercuric chloride inactivates purified hæmolysin which can be restored by treatment with hydrogen sulphide and reduced glutathione.

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THE EARLY STAGES OF *MUSCA INFERIOR* STEIN.

BY

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AND

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(*Fly Research Inquiry, Indian Research Fund Association, School of Tropical Medicine, Calcutta.*)

EXCEPT for a figure of the egg in a recent paper by Ch'i Ho (1938), the early stages of *Musca inferior* have not been described (Patton, 1937). In the present communication, the ovarian egg, third stage larva, and puparium, are described.

This is a large hæmatophagous species which feeds on cattle, and is easily recognized by the hairs on the squama, a feature not possessed by any other oriental species of *Musca* (Patton, *loc. cit.*). It breeds in isolated patches of cow-dung. The following table gives data of flies bred out from larvæ collected on the 'Maidan' in Calcutta :—

Date.	Number per patch.	Remarks.
8th June, 1938 ..	3	
13th July, 1938 ..	12	
21st September, 1938 ..	7	
14th October, 1938 ..	4	
19th October, 1938 ..	77	Six patches of cow-dung together.

J, MR

14

EXPLANATION OF PLATE XL.

All figures, except Fig. (*f*), refer to *M. inferior.*

Fig. (*a*)—Egg; Fig. (*b*)—Abdominal inter-segmental spinal bands of the larva (right lateral view); Fig. (*c*)—Eighth abdominal segment of the larva (left lateral view); Fig. (*d*)—Anal plate of the larva; Fig. (*e*)—Posterior spiracles of the larva; Fig. (*f*)—Posterior spiracles of the larva of *M. nebulo* [drawn to the same scale as Fig. (*f*)]; Fig. (*g*)—Cephalopharyngeal skeleton of the larva (left lateral view); Fig. (*h*)—Pupal spiracles; Fig. (*j*)—Intra-pupal spiracles; Fig. (*k*)—Puparium.

an. pap.—anal papillæ; *an. pl.*—anal plate; *c.*—cicatrix; *c.p.*—coronoid process; *d.b.*—dorsal bridge; *d.s.*—dental sclerite; *i.p.s.*—intra-pupal spiracle; *l.h.r.* and *l.h.l.*—right and left lateral hooks respectively; *l.p.*—lateral plate; *m.s.*—intermediate sclerite; *p.*—peritreme; *p.gl.*—opening of a peristigmatic gland; *ph.r.*—pharyngeal ridges; *ph.s.*—pharyngeal sclerite; *post. sp.*—posterior spiracles; *pup. sp.*—pupal spiracles; *st.*—spiracular slits; *v.r.*—ventral ridge; 1°*b.*—primary inter-segmental band of spines; 2°*b.*—secondary band.

PLATE XL.

(a)

(b) 2°b 1°b. v.r.

(c) post. sp. an. pl. an. pap

(d)

(e) st. p.gl. -c ---p.

(f)

(g) d.b. l.p. ph.s. c.p. l.h.l m.s. d.s. l.h.r ph.r 0·5 mm

(h) i.p.s.

(j) 0·1mm

(k) pup sp 1 mm
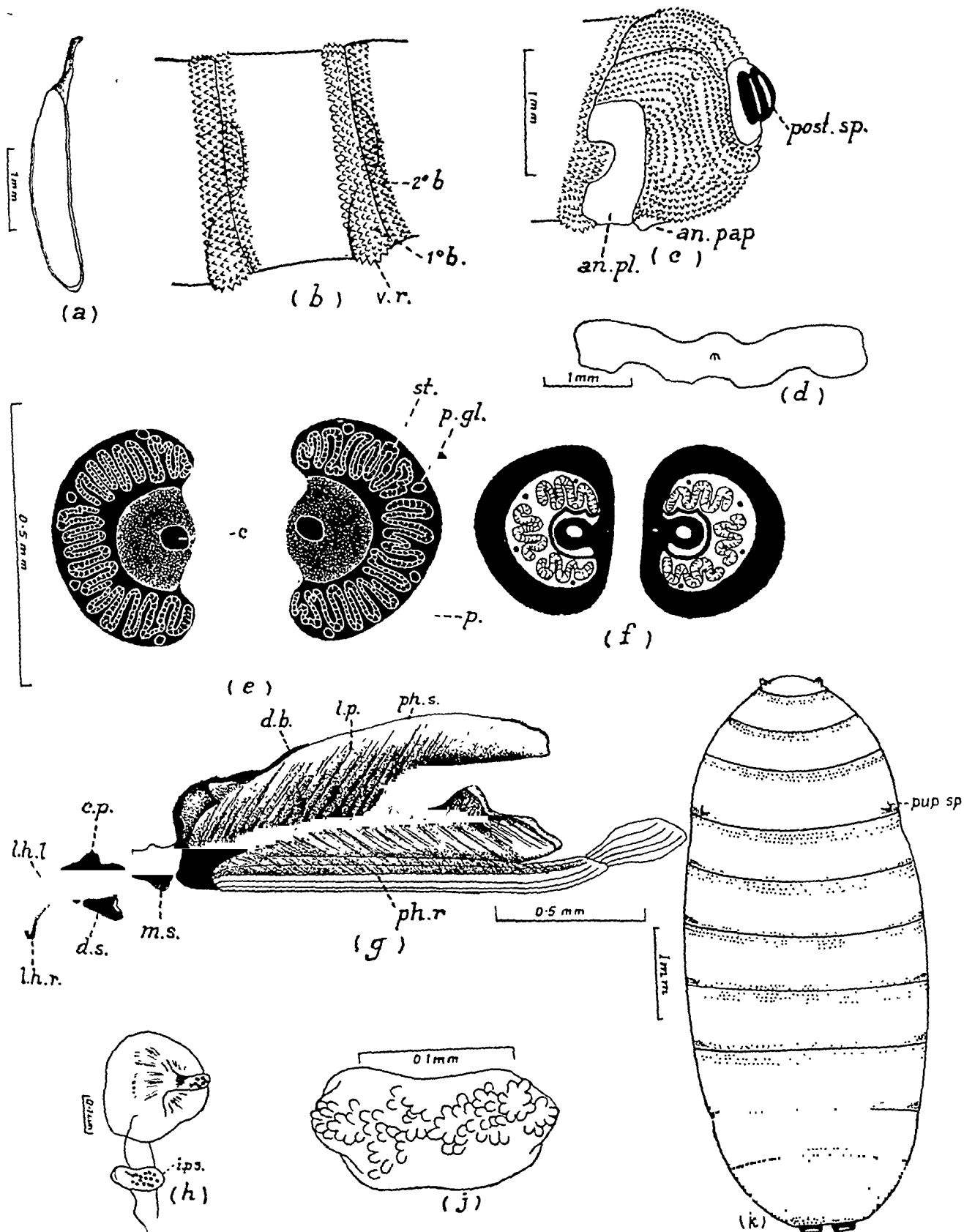
Several collections of muscoid larvæ were made in March, April, and May 1939, but not a single specimen of this species was obtained.

Material and methods.—The eggs were obtained by dissection from gravid females, and measured in normal saline solution. In describing the other structures we have mainly followed Keilin (1917) and Muirhead Thomson (1937), and have dealt only with those characters which are useful for systematic purposes, and those which throw some light on the feeding habits.

Egg [Plate XL, fig. (*a*)].

The egg is stalked as in *M. spinohumera* and *M. pattoni* which also breed in cow-dung. It measures 3·2 mm. in length and 0·47 mm. in breadth. The stalk is less than one-quarter of the total egg-length.

The number of eggs produced per batch is about 22 (wild flies). A laboratory-bred fly, sustained by feeding on a rabbit, sugar, and water, produced seventeen eggs.

Third stage larva.

The amphineustic larva conforms to the *Musca* type (Hewitt, 1914), but is relatively large ; the length is about 15 mm., and its colour is creamy white.

The colourless inter-segmental spines are well developed for a *Musca* larva. They form complete belts at the anterior margin of all the segments and cover the ventral pads. In addition to these primary bands, there are secondary bands placed at the posterior margins of the first six abdominal segments [Plate XL, fig. (*b*)]. The spines of the secondary bands are most prominent laterally and in the mid-dorsal line of the posterior segments. The primary bands and traces of the secondary bands are also present in the larva of *M. pattoni* var.*, but not in the larvæ of the common house-frequenting species, *M. domestica*, *M. vicina*, *M. nebulo*, *M. sorbens*, and *M. yerburyi*. *M. vetustissima* has the spined lateral ridges of the secondary bands.

The eighth abdominal segment [Plate XL, fig. (*c*)] is covered with spines. The anal plate [Fig. (*c*)—*an. pl.* and Fig. (*d*)] is devoid of spines, and has long, broad wings, whereas in *M. domestica* (Muirhead Thomson, *loc. cit.*), *M. vicina*, and *M. nebulo* the wings are not developed at all, and in *M. sorbens* the wings are short, narrow, and tapering.

Anterior spiracles. The number of papillæ is commonly twelve, though as in other larvæ there may be slight variation.

Posterior spiracles [Plate XL, fig. (*e*)]. The spiracular plates are very large ; they measure 0·51 mm. in height and 0·35 mm. in width. The corresponding figures for *M. nebulo* [Fig. (*f*)] are 0·30 mm. and 2·4 mm., respectively. The distance between the plates is about one-fifth the width of a plate. The inner margin may be broadly indented, so that the plate is kidney-shaped. The peritreme (*p*) is densely chitinized. The stigmata or spiracular slits (*st.*), three in number, are narrow and very convoluted.

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*Hairs present on the supra-squamal ridge.

Cephalopharyngeal skeleton [Plate XL, fig. (*g*)]. The mandibular sclerites or lateral hooks (*l.h.r.*, *l.h.l.*) are curved and narrow, especially the left, which, as is usual in *Musca,* is shorter than the blunt, right hook, but the disparity is not so pronounced as in the *domestica* group. The coronoid processes (*c.p.*) are well developed. The dental sclerites (*d.s.*) do not form a median ventral arch. The intermediate sclerite (*m.s.*) is separate from the pharyngeal sclerite. The latter (*ph.s.*) has the posterior emarginations. The lateral plates (*l.p.*) are unevenly chitinized, the densest parts being the anterior margin and the dorsal bridge (*d.b.*). The ventral plate is provided with longitudinal ridges (*ph.r.*). Thus, from the presence of pharyngeal ridges, the absence of accessory sclerites, and the loose articulation of the intermediate piece, the larva is shown to be saprophagous in its feeding habits.

Puparium [Plate XL, fig. (*k*)].

The dark-brown puparium measures 6·5 mm. in length and 3 mm. in breadth. The descriptions of the larval structures which are present in the puparium apply here, except that the parts are now strongly chitinized and somewhat shrivelled.

The pupal spiracles [Fig. (*h*) and Fig. (*k*)—*pup. sp.*] are present, projecting dorso-laterally from the posterior margin of the first abdominal segment. The papillæ of the intra-pupal spiracles [Fig. (*h*)—*i.p.s.* and Fig. (*j*)] are arranged in a longitudinal pattern consisting of about nine groups.

Eclosion is by the usual splits.

The pupa is parasitized by *Dirhinus pachycerus* Masi (Ord. Hymenoptera, Sup.-fam. Chalcidoidea, Fam. Chalcididæ).

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A STUDY OF SOME VIRULENT AND AVIRULENT STRAINS OF *PASTEURELLA PESTIS*.

BY

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AND

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THE phenomena of dissociation of *Pasteurella pestis* have not yet been fully worked out and the findings of workers on the subject differ. Wu Lien-Teh (1936) has given a very comprehensive review of the diverse conclusions of the various workers and concludes that further studies on this subject, including exhaustive investigations of quite fresh material, are called for. As recently-isolated as well as old cultures of *Pasteurella pestis* are available at the Haffkine Institute we undertook this investigation to clear the position, if possible.

The preliminary study consisted of noting the colonial morphology, morphology of the organisms, growth and stability in broth, etc., of individual strains.

EXPERIMENTAL METHODS.

Media : blood agar.—In order to study colonial morphology and variation, five per cent superimposed rabbit-blood agar was used. The slopes were inoculated from appropriate dilutions of the growth, so as to give widely separated colonies between 20 and 30 for each. These were incubated in the humid incubator at 37°C. for 72 hours and in some cases at room temperature (27°C. to 29°C.) as well. A binocular dissecting microscope (Bausch and Lomb) was employed in studying the colonial morphology and the smears from individual types of colonies were examined after staining with carbol-thionin solution.

Broth.—Haffkine's acid-digest broth pH 6·8 was utilized for noting the characteristic type of growth, and the morphology of the organisms by staining as well as under the dark-ground illumination. The broth tubes after inoculation were

incubated at 37°C. for 48 hours and in some cases at room temperature (27°C. to 29°C.) as well.

Normal saline containing 0·85 per cent sodium chloride was used for 'salt stability' tests. One millimetre loopful of the growth at 37°C. on blood agar was emulsified in 3 c.c. of normal saline distributed in test-tubes 4″ × 0·5″. These tubes were then incubated at 37°C. and observed after 24 and 48 hours.

The preliminary work in this series was confined to the strains 120/5H virulent and avirulent. This strain was originally isolated from the heart blood of a septi-cæmic case during the 1932 plague epidemic of Hyderabad. In 1939, for the present study, this strain was passaged through Madras rats ten times and a fully virulent form of the strain obtained. The avirulent form of this strain was obtained by Sokhey (1936) through a series of sub-cultures on 'acid-digest agar' incubated at 37°C.

Colony appearance.—Two distinct types of colonies were obtained from these sub-cultures. The virulent culture of 120/5H produced uniform small translucent dew-drop-like colonies with a shiny pearl-like appearance. They were convex, rounded with a smooth glistening surface. These we have designated as 'smooth' though we do not attach the same significance as in the case of other bacteria.

The avirulent culture of 120/5H produced comparatively large-sized colonies with a pin-pricked, sometimes rugose, surface and irregular, broken, or serrated margins.

Morphology of the organisms.—The 'smooth' colonies consisted of uniform oblong bacilli (Plate XLI, fig. 1), while the 'rough' ones were composed of long forms of varying sizes as shown in the microphotographs (Plate XLI, fig. 2).

Carbohydrate and protein reaction.—The carbohydrate and the protein reactions of the virulent and avirulent cultures showed appreciably no differences. It may, however, be observed that under identical conditions of inoculation and incubation the virulent cultures exhibited more marked activity in fermenting the sugars, while the avirulent ones were sluggish.

The protein reactions were the same in both cases, the virulent and the avirulent forms giving positive nitrite reaction.

Type of growth in broth.—The virulent growth in broth was uniformly turbid, while the avirulent one gave a granular deposit at the bottom and sides with a comparatively clear supernatant fluid. On shaking the granular growth distributed itself in the fluid in clumps which again settled down after a short time. The virulent culture was 'salt stable', while the avirulent was 'salt sensitive'.

APPEARANCE UNDER DARK-GROUND ILLUMINATION.

Broth cultures.—The virulent growth at 37°C. exhibited chain formation, the individual members composing the chain retaining the normal ovoid forms. In the avirulent growth the chains were much longer, the individual organisms thinner and of varying lengths.

Agar growth.—Agar growth showed no chain formation in either strain at 37°C. or 27°C. ; the organisms of the avirulent strain were long and slender, while those of the virulent strain were ovoid and stouter.

Sub-cultures from individual colonies.—To ascertain whether the type of colonies observed were capable of breeding true, isolated colonies were picked up, emulsified, and cultured on blood agar to get discrete colonies.

The avirulent strain reproduced a majority of colonies similar to its parent at 37°C. as well as at room temperature (27°C. to 29°C.). The virulent on the other hand gave a majority of 'smooth' colonies at 37°C. and a larger proportion of 'rough' colonies at the room temperature.

The main characters of the virulent and avirulent strains of 120/5H may be summarized as follows :—

	Virulent.	Avirulent.
1.	Majority of the colonies small, 'smooth', transparent, convex, glistening, and with regular margins.	Majority of the colonies fairly large with pin-pricked or rugose surface, flat, opaque with serrated or broken peripheries.
2.	Organisms normal being ovoid.	Organisms long and thin exhibiting pleomorphism.
3.	Uniformly homogeneous growth in broth.	Granular growth with deposit at bottom and sides and a fairly clear fluid at the top.
4.	Salt stable.	Salt sensitive.
5.	Breeds a majority of 'smooth' colonies at 27°C. as well as at 37°C. sub-cultures.	Breeds a majority of rough colonies at 27°C. as well as at 37°C. sub-cultures.

Morphology of primary colonies.—The study was extended to the primary cultures received from an epidemic area (Bettiah) in 1937-38. As a routine, blood from a plague-infected case is spread on standard agar slopes, these slopes being paraffined, capped, well packed, and posted to Bombay. At the time of arrival *Pasteurella pestis* colonies are apparent ; the cultures being about four days old, growing in transit. A similar procedure is followed in the bubo cultures, the material from the punctured bubo being used for the inoculation of the tubes.

The cultures investigated were from the blood and buboes of septicæmic cases. As a result of the study of such cultures we came across three distinct types of colonies, leaving out the minor differences.

A photograph of the important types of colonies that we have been able to observe in these primary cultures is shown.

The primary cultures from the blood as a rule presented the following distinct types of colonies : a small, a large, and an intermediate medium-sized variety. The small colonies were smooth, translucent with shiny surface (Diagrams 1, 3, 6, 7, and 9, Plate XLII). The large colonies were 'rough', with irregular, broken edges,

pin-pricked, rugose, or convoluted surface or at times even circumvallate (Diagrams 2, 4, 10, 11, and 14, Plate XLII). The intermediate type consisted of medium-sized colonies with irregular margins and ruffled surface, sometimes showing a nippling in the centre or gemmation, or at times appearing like a blob of pus (Diagrams 8, 12, and 13, Plate XLII).

The bubo cultures presented two distinct types of colonies, one type consisting of small, convex 'smooth' rounded colonies, with well-defined margins, and the other consisting of large, 'rough' surfaced whitish protruding colonies. In colonial characteristics, morphology of the organisms, nature of growth in broth, salt stability, and other characteristics the small 'smooth' colonies resembled those obtained from the virulent cultures of 120/5H, and the large whitish 'rough' colonies were similar to the ones obtained from the avirulent strain. The intermediate type microscopically showed normal type of ovoid organisms as well as marked pleomorphism, and as its name implies, seemed to be a middle stage between the two colonies.

To ascertain whether these and other strains stocked at the Haffkine Institute were capable of breeding true, sub-cultures of individual type of colonies were made as described above. As a general rule the room-temperature growth of the virulent strain gave a small percentage of 'rough' colonies but the 37°C. growth propagated almost all 'smooth' colonies, though the predominance of the 'rough' or the 'smooth' type at the respective temperature varied from strain to strain.

Tables A and B given below show the approximate percentages of the 'smooth' and 'rough' colonies which were obtained on sub-culturing a few of the stock strains of *Pasteurella pestis* at room temperature and 37°C.

TABLE A.

Incubation at room temperature (27°C. to 29°C.).

Strains.	'Smooth'. Per cent.	'Rough'. Per cent.
Vimlabai	27	73
34/B	30	70
35/B	60	40
36/H	58	42
120/5H (10th passage) ..	85	15
*120/5H avirulent	2	98
*P „	10	90
*Q „	6	94
55/H „	75	25

* Rendered avirulent by Sokhey (1936) through repeated sub-culture at 37°C.

PLATE XLI.
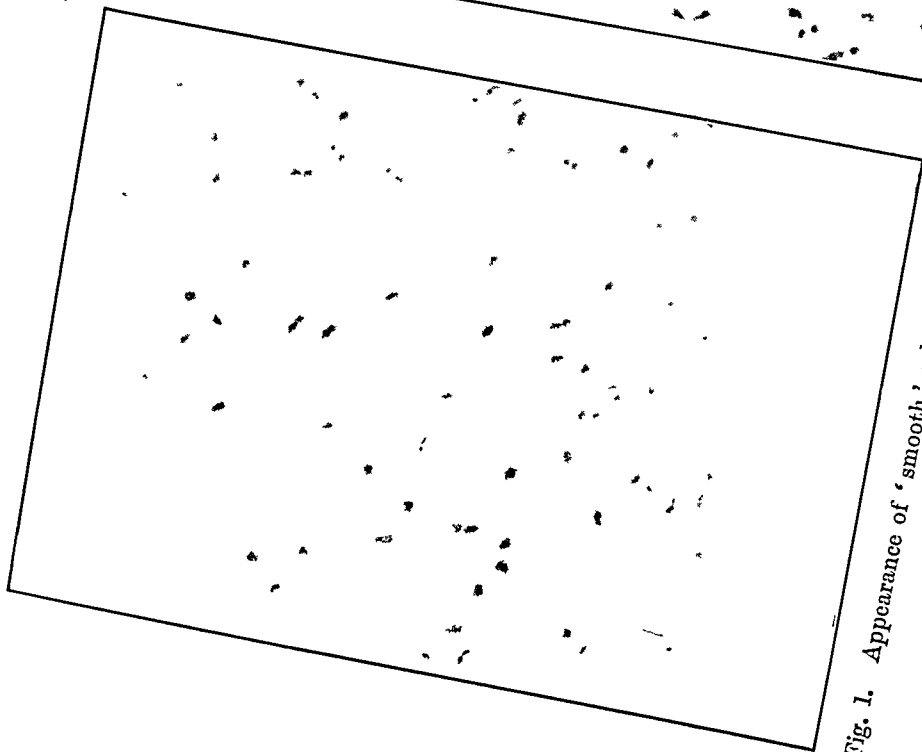
Fig. 1. Appearance of 'smooth' colony organisms.
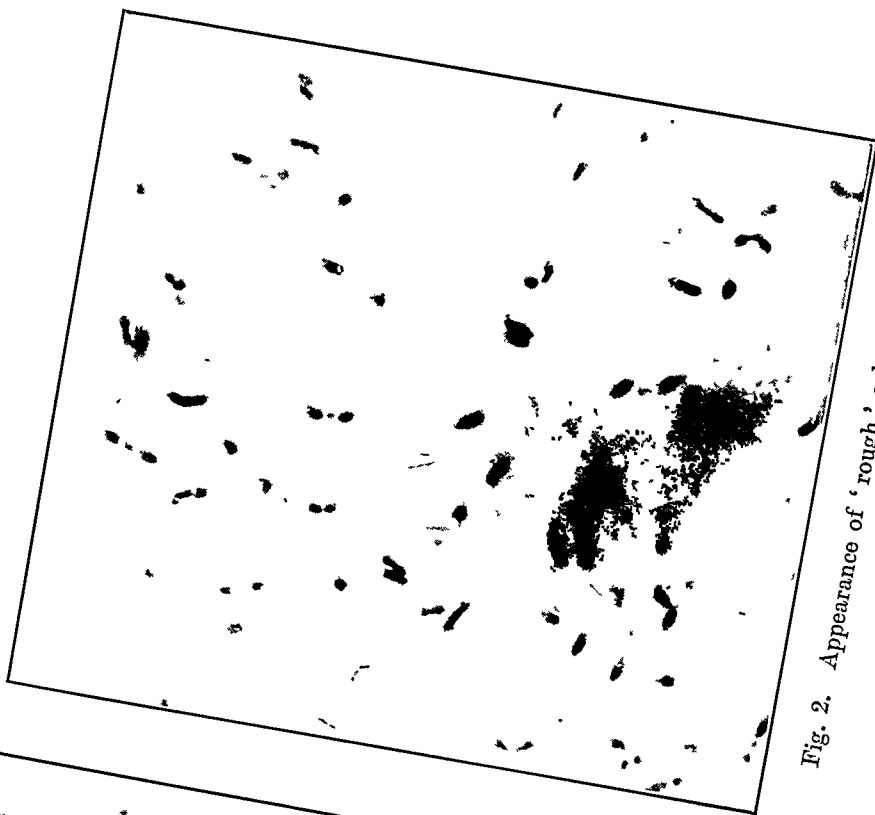
Fig. 2. Appearance of 'rough' colony organisms.
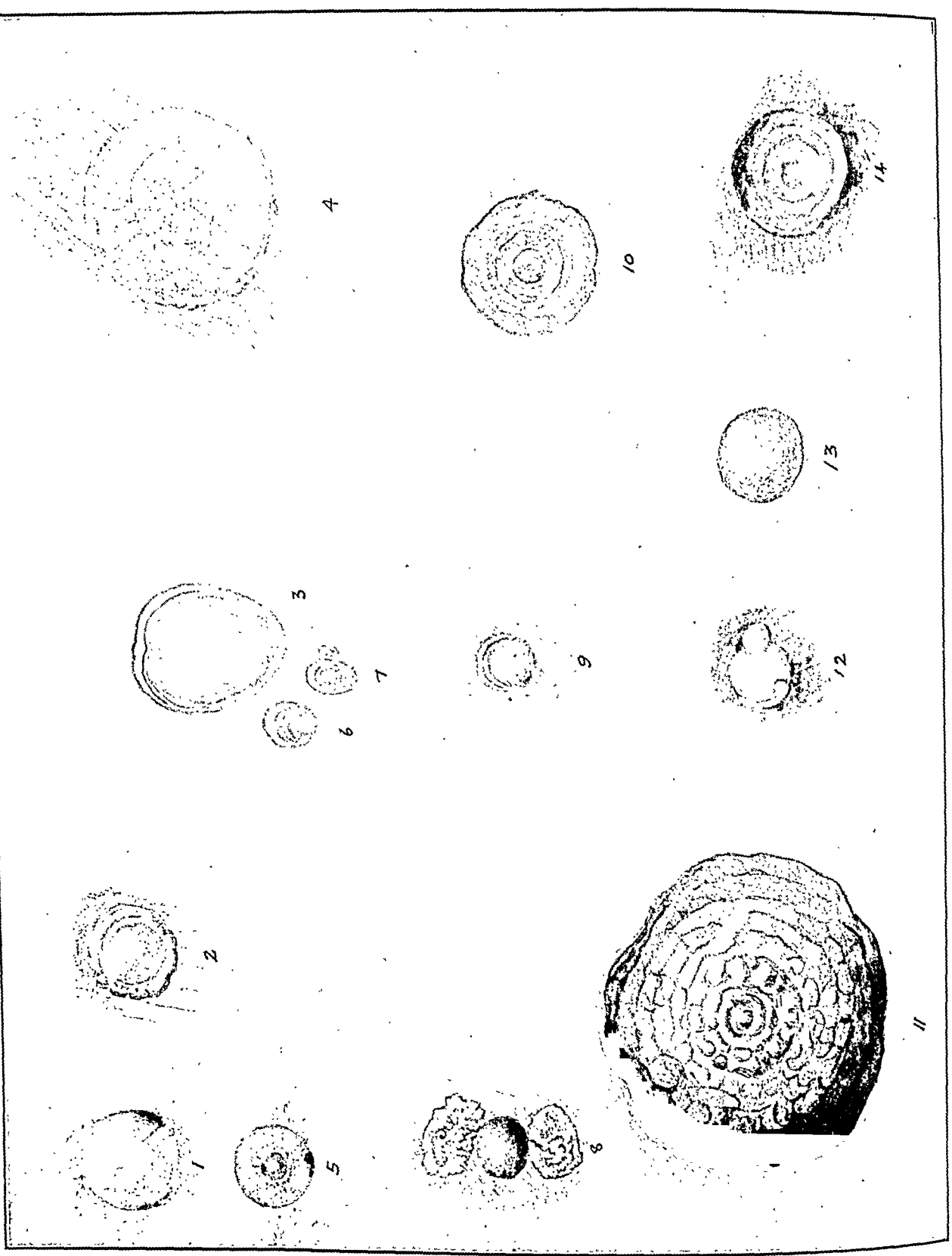
Colonial variations of *Pasteurella pestis* in the primary cultures. For descriptions of the types of colonies see text.

TABLE B.

Incubation at 37°C.

Strains.	'Smooth'. Per cent.	'Rough'. Per cent.
Vimlabai	65	35
34/B	78	22
35/B	80	20
36/H	55	45
120/5H (10th passage)	98	2
*120/5H avirulent	8	92
*P ,,	12	88
*Q ,,	10	90
55/H	92	8

* Rendered avirulent by Sokhey (1936) through repeated sub-culture at 37°C.

It would be observed that no virulent strain gave cent per cent 'smooth' colonies at 37°C. or an avirulent strain cent per cent 'rough' colonies at 27°C.

SEROLOGICAL DIFFERENTIATION OF THE STRAINS.

The presence of a soluble material in the washings of the growth of *Pasteurella pestis*, especially when grown at 37°C., has been observed. This substance is practically obtained by the same technique as the 'envelope substance' of Schütze (1932) and we have designated as the 'soluble substance'. One of us (Wats, 1938) worked with a lysate of the agar-growth cultures of *Pasteurella pestis* for titrating the anti-plague sera prepared at this Institute. It was noted that the lysates of some strains were very poor for the precipitin reaction as compared to others. An attempt was made to correlate this finding with the characteristics of 'rough' and 'smooth' colonies noticed above.

EXPERIMENTAL METHODS.

Preparation of the soluble substance solution.—One mm. loopful of the culture to be tested was inoculated into acid-digest broth and incubated at 37°C. for 48 hours. Roux bottles containing acid-digest agar were inoculated with this broth culture and incubated at 37°C. for 72 hours. The resulting growth was washed with about 15 c.c. of normal saline, well shaken by hand, and allowed to stand at room temperature for two hours. The tubes were then heated in a water-bath (55°C.) for 30 minutes, centrifugalized to obtain a clear supernatant fluid.

Antiplague serum.—For this test polyvalent antiplague serum (No. 16) was employed. This serum was prepared at the Haffkine Institute by immunizing horses with 37°C. live growth of 120/5H (avirulent) and later with other mixed strains. The strains used for this particular horse are 120/5H (avirulent and virulent), Vimlabai, Strain Nos. 34 to 39 and Nos. 49 to 56.

Technique of the test.—The method of optimal proportions as described by Dean and Webb (1928) was used for putting up the test. To decide the appropriate dilutions of the serum to be used, a range of antiserum and S. S. solution were put and finally 1/200 dilution of the antiplague serum was selected, being most suitable for comparative purposes.

To enable us to read the results in all the tubes simultaneously, strips of aluminium sheeting $\frac{3}{4}''$ broad with holes corresponding to the rows in the agglutination rack were loosely fitted to the metal rack. Lipped agglutination tubes of a uniform size (4 cm. × 0·5 cm.) were fitted through the aluminium strips in the agglutination stands. Into each tube 0·5 c.c. of the appropriate dilution of the S. S. solution was poured and 0·5 c.c. of the 1/200 dilution of the antiserum added. The dilutions of the S. S. solution were 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128. The appropriate controls with normal saline and normal horse serum were put up with each series of experiments. The tubes were heated in a water-bath (55°C.) for two hours. The readings were noted after every 15 minutes and the dilution of the S. S. solution showing the optimal flocculation noted. It is evident that as the serum dilution is constant, the higher the dilution of the S. S. solution required to give the optimal flocculation, the higher the soluble substance contents of the washings.

The Table summarizes the results of the study of the types of growths on solid and in liquid media, the morphology of the organisms and the S. S. titre. The cultures were cultivated at 37°C. except where otherwise stated :—

TABLE.

Strains.	Types of colonies.	Microscopic appearance.	Types of growth in broth.	S. S. titre.
53/H	Medium-sized intermediate.	Pale staining plump organisms rather longish.	Partly homogeneous	1/16
Java	Large flat and 'rough'.	Pale staining ovoid forms.	Granular ..	1/4
Egypt ..	Large 'rough' as well as circumvallate.	Deep staining longish organisms.	Fairly homogeneous	1/16
S. Africa ..	Small translucent dew-drop-like and large whitish opaque convex.	Organisms long and thin many thready forms.	do.	1/4

TABLE—*concld.*

Strains.	Types of colonies.	Microscopic appearance.	Types of growth in broth.	S. S. titre.
49/Bit ..	Small 'smooth' shiny pearl-like.	Uniformly stained ovoid forms.	Homogeneous ..	1/2
35/B	Large and small 'smooth'.	Pale staining ovoid organisms.	do. ..	1/8
36/H	Small uniform sized 'smooth'.	Thready forms ..	Granular growth ..	1/4
55/H	Small 'smooth' shiny.	do. ..	do. ..	1/64
56/H	Large 'smooth' ..	do. ..	do. ..	1/8
40/M	Small 'smooth' and pearly.	Short ovoid forms	Homogeneous	1/32
39/B	Uniform sized 'smooth' glistening.	Small plump and ovoid forms.	do. ..	1/8
34/B	Small flat 'smooth' glistening with a metallic lustre.	Faintly staining small plumpy forms.	do. ..	1/8
1. virulent ..	Large 'smooth' shiny.	Very pleomorphic varying from ovoid to stout rod-shaped forms.	Fairly homogeneous	1/4
I. avirulent ..	Large 'rough' ..	Very long and thin forms, few thready.	Granular ..	*Nil.*
120/5H ..	Small dew-drop-like translucent shiny.	Bipolar staining uniform sized organisms.	Homogeneous ..	1/64
120/5H avirulent	Large 'rough' ..	Uniformly stained rod-shaped organisms.	Granular growth with deposit at bottom and sides.	1/64
120/5H avirulent (27°C. growth).	Large 'rough' ..	Uniformly stained long thin forms.	do. ..	*Nil.*
P. avirulent ..	'Rough' circumvallate, rather large.	Almost all the forms typically thready.	do. ..	*Nil.*
Q. avirulent ..	'Rough' and circumvallate.	Long thready forms, few slender forms.	do. ..	1/32
Madagascar, E. V. (Avirulent strain used for manufacturing live vaccine).	Small and large 'smooth'.	Ovoid and few thready forms.	Homogeneous ..	1/64

DISCUSSION.

Though no hard and fast rule can be laid down in the case of *Pasteurella pestis* it is apparent that there is undoubtedly a correlation between the colonial morphology, the morphology of the organisms, and the type of growth in the broth. The ' smooth ' colonies always seem to show a normal type of organism, plump and ovoid, and a fairly homogeneous growth in broth. On the contrary in the ' rough ' colonies the organisms appear long, thin and sometimes thready, with a granular type of growth in broth.

Soluble substance.—The presence or absence of the S. S. is obviously not dependent on the production of the ' smooth ' or ' rough ' colonies by a strain, but as a general rule it may be stated that a strain producing majority of ' rough ' colonies is likely to be poor in the S. S. value.

Relationship to virulence.—It is also evident from the Table that the presence or absence of the S. S. has no relation to the virulence or non-virulence of the strains studied as avirulent strains, e.g., Madagascar, Java, Egypt, 120/5H, Q, etc., show its presence in varying proportions. It is, however, to be noted that all virulent strains inevitably produce it though the S. S. titre varies from strain to strain.

The significance of the soluble substance.—Sokhey (1936) tested the relative value of the antiplague vaccines made from heat-killed virulent and live avirulent cultures of 120/5H. These vaccines were prepared from agar-grown organisms incubated at 27°C. In subsequent work he compared the value of the live avirulent vaccines with heat-killed virulent strain vaccine prepared from the agar growth of 120/5H at 37°C. From his results it is evident that the 27°C. agar growth of avirulent strain of 120/5H gave a very poor vaccine, whereas the 37°C. agar of both virulent as well as avirulent gave potent vaccines in both cases—avirulent being if anything better than the virulent one. If we refer to the Table above, it will be seen that the S. S. titre of 120/5H avirulent is *nil* in the case of 27°C. growth, while on the contrary the 37°C. growth of the avirulent as well as virulent cultures show an equally high titre of the same. If the presence or absence of the S. S. can be used as an indication of the protective value of the strain we should expect the I. avirulent strain to give a very poor vaccine even at 37°C., as the S. S. titre is *nil*. That this is so, is evident from Sokhey's findings (Sokhey, 1937). He states that the antigenic value of this strain has entirely disappeared during the process of rendering it avirulent by repeated sub-culturing at 37°C. Further work is being carried out to develop this point.

SUMMARY AND CONCLUSIONS.

1. The colonial morphology of *Pasteurella pestis* has been studied from several virulent and avirulent strains. The production of the types of colonies on sub-culture, as well as the morphology of the organism and the types of growth in broth, have been investigated.

2. The soluble substance in the supernatant fluid of the heated washings of the agar-grown cultures has been estimated in the various strains and its significance discussed.

3. The titre of the soluble substance seems to indicate the antigenic value of a strain.

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FURTHER STUDIES ON THE NUTRITION OF THE PLAGUE BACILLUS : THE RÔLE OF HÆMATIN AND OTHER COMPOUNDS.

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INTRODUCTION.

In the study of bacterial nutrition the concept of growth factors is of much importance. A growth factor is distinguished from the numerous other compounds that a micro-organism may utilize in the course of its growth by the fact that in its absence no growth occurs. Its indispensability is an expression of the more significant fact which was first pointed out for bacteria by Knight (1936) that the organism has lost the ability to synthesize the molecular structure represented by the growth factor. In the absence of a deeper insight into the reactions taking place in the cell during growth this view, till now, has adequately summed up the function of the growth factor in nutrition. There are two types of bacterial growth factors : (1) simpler molecules, such as the amino acids and (2) more complex molecules, such as thiamin, nicotinic acid, etc. The latter type of compounds have more recently acquired a new interest on account of the discovery that they have a universal and fundamental rôle in cellular respiration. It has been found that some of the complex growth factors are identical with animal vitamins ; and further that these compounds owe their importance both in the animal and in the bacterial cell to their function as co-enzymes in enzymatic oxidation systems. Co-enzymes differ from enzymes in being heat-stable compounds of lower molecular weights and in functioning as hydrogen-carriers in oxidation-reduction reactions (Baumann and Stare, 1939 ; Barron, 1939). The recent work on four complex bacterial growth factors, which figure in the experiments reported below, can be briefly summarized as follows :—

Thiamin.—Thiamin or aneurin is identical with vitamin B_1. Knight (1937) and Knight and McIlwain (1938) have shown that thiamin is a growth factor for *Staphylococcus aureus.* A clue to the function of this vitamin in metabolism was

provided by Lohmann and Schüster (1937) when they showed that thiamin pyrophosphate is the co-enzyme of the enzyme carboxylase of yeast which breaks down pyruvic acid to acetaldehyde and carbon dioxide. These authors suggested that the pyrophosphate is the active form of vitamin B_1 in animal tissues; and this hypothesis has now been proved by Banga, Ochoa and Peters (1939). Lipmann (1937) has shown that the pyrophosphate is necessary (but not the free vitamin) for the oxidative de-carboxylation and the dismutation of pyruvic acid by acetone preparations of *Bacterium delbruckii*. Hills (1938) has found that thiamin is necessary for the oxidation of this substrate by *Staph. aureus*. And more recently, Barron and Lyman (1939) have found a similar rôle for this growth factor and its pyrophosphate in *staphylococci, gonococci*, and hæmolytic *streptococci*.

Phosphopyridine nucleotides.—The two compounds di- and triphosphopyridine nucleotides are the co-enzymes I and II, respectively, of fermentation and de-hydrogenations. Lwoff and Lwoff (1937a) have shown that *Hæmophilus parainfluenzæ* cannot grow unless one of these is provided and that either of them is identical with the V-factor. They have demonstrated the close dependence of oxygen uptake and methylene-blue reduction of the bacteria on the amount of nucleotides available. The elucidation of the structure of these compounds and their function in de-hydrogenations is due to Warburg who has shown that they consist of one molecule of adenine and one of β-nicotinic-acid amide combined with 2 or 3 molecules of phosphoric acid and two of ribose; and that by being alternately reduced and oxidized they serve to carry hydrogen from and to substrate molecules (Warburg, Christian and Griese, 1935; Warburg and Christian, 1936a, b).

Nicotinic acid.—Nicotinic acid is the anti-pellagra vitamin. Knight (1937) was the first to report on the necessity of this compound for the growth of *Staph. aureus*. Its importance for the cell is supposed to be due to its occurrence (as nicotinic-acid amide) in the molecule of the phosphopyridine nucleotides.

Hæmatin.—Hæmatin is identical with the X-factor for *Hæmophilus influenzæ*. The large amount of older work on this growth factor, which has been summarized by Knight (1936), showed that X-activity is associated with peroxidase and catalase activity, and that the compound is necessary for ærobic mode of life but not for anærobic existence. In a more recent work Lwoff and Lwoff (1937b) have investigated the necessity of hæmatin as a growth factor for *Hæmophilus* and have further shown that it is necessary to maintain the level of respiration. They have suggested that hæmatin is required for the formation of cellular oxidation systems containing this molecule as the prosthetic group, such as cytochrome, cytochrome-oxidase, catalase, and peroxidase.

In a previous paper (Rao, 1939) it has been shown that, while only three amino acids—proline, phenylalanine, and cystine—are indispensable for the growth of the plague bacillus the more complex type of growth factors are not essential. The latter type of compounds, therefore, can be considered to be structurally dispensable. However, in view of their function as cellular catalysts they should possess a *stimulatory* activity on the metabolism and the growth of the organism. This question has been tested experimentally in the present paper with the help

of six compounds known to function as growth factors or as co-enzymes, viz., (1) hæmatin, (2) cozymase or diphosphopyridine nucleotide, (3) thiamin, (4) nicotinic acid, (5) isoalloxazine-adenine-dinucleotide, and (6) β-alanine. Three series of experiments are described :—

I. The stimulation of the growth of the organism by the compounds in a chemically defined medium.

II. The stimulatory effect of the compounds on the respiration of growing cultures of the organism.

III. The stimulatory effect of the compounds on some of the oxidations catalysed by washed suspensions of the bacillus.

TECHNIQUE.

An amino-acid medium for growth tests.—For testing the stimulatory action of the selected compounds on the growth of the bacillus a medium of known chemical composition is necessary. For this purpose an amino-acid mixture based on the similar medium previously described (Rao, 1939), but containing twelve amino acids was designed. The composition of the new medium is given in Table I. It has been enriched considerably by the inclusion of several amino acids known to

TABLE I.

An amino-acid medium for the plague bacillus.

Constituent.	Concentration, mg./500 c.c.
dl-proline	70
dl-phenylalanine	107
l-cystine	40
dl-alanine	60
d-glutamic acid	50
glycine	50
l-tyrosine	64
dl-serine	70
dl-isoleucine	65
dl-leucine	90
dl-methionine	75
dl-valine	65
Bacterial ash equivalent to ..	25 mg. bacteria.
M/30 phosphate buffer at pH 7·4 ..	50 c.c.

be utilized by the organism (Rao, 1940), and by the omission of those which are unattacked. The concentration of the natural optical isomer of each amino acid is M/1500 ; while the concentration of glycine is M/750. Instead of depending on the impurities contained in phosphate and other constituents for the supply of indispensable inorganic elements (of which we have no knowledge at present) the ash of the organism itself was prepared and added. The addition of special carbon sources, such as glucose and lactic acid, was found to cause the organism to grow in clumps, particularly when the medium was poorly buffered, so that observation of growth by turbidity was difficult.

The test compounds.—The details of preparation, purity, etc., of the compounds whose activity was to be tested are as follows :—

1. *Cozymase.*—This compound was prepared by the method of Ohlmeyer (1938) from dry beer yeast. Its purity was about 0·2.

2. *Hæmatin.*—A sample supplied by Merck was used. A standard solution in dilute NaOH was prepared. The compound remains in solution only when the pH of the medium is on the alkaline side of pH 7.

3. *Thiamin.*—A crystalline synthetic specimen by Bayer was used.

4. *Nicotinic acid.*—This compound was synthesized according to the method described in ' Organic Syntheses ' (Gilman, 1932).

5. *Isoalloxazine-adenine-dinucleotide.*—This compound is the co-enzyme of *d*-amino-acid oxidase of animal tissues and the diaphorase of cellular oxidation systems. It is the prosthetic group of Warburg's yellow ferments. Its riboflavin moiety is identical with vitamin B_2. The dinucleotide was isolated from Marmite, which was found to be a richer and a more convenient source than the usual yeast maceration extract, by the method of Warburg and Christian (1938) for the latter, viz., extraction with phenol, precipitation as the silver salt, conversion into the barium salt, and a re-extraction with pure phenol. A watery solution containing the compound from 1·0 g. Marmite in 1·0 c.c. was finally prepared. This compound is referred to as flavin below.

6. *β-alanine.*—This was a synthetic sample supplied by Schüchardt.

Conduction of growth tests.—The tests were designed to observe the stimulatory effect of the above compounds on the growth of the organism in the amino-acid medium. Into each specially cleaned thick-walled test-tube 10 c.c. of the amino-acid mixture were added along with the concentrations of the test compounds given in Table II. The tubes were sterilized at 120°C. for 20 minutes. All the compounds are fairly stable at this temperature except possibly cozymase. The tubes containing the latter were sterilized at 110°C. for 5 minutes. The amino-acid mixture contains all the factors essential for the growth of the organism. The only activity expected from the added test compounds was, therefore, a further stimulation of the growth. For estimating the degree of stimulation a simple procedure similar to that usually employed in nutritional studies was followed. Observations were made on the ability of the added compound to shorten the lag period of growth, or in other words, to initiate an earlier multiplication of the added

inoculum over that of the control consisting of the amino-acid mixture only. The lag phase is unusually long in the case of the plague bacillus in the laboratory media (except blood agar) and is even longer in the amino-acid medium. The reduction in the length of this period, therefore, gives a reliable indication of the stimulation exercised by the test compound. The tubes were inoculated with a well-washed suspension of strain ' 120/5 H, virulent '. (This strain has been already submitted to detailed study in the previous papers). The bacillus was grown on agar slopes, as described before, collected in saline and washed on the centrifuge six times with large batches of saline, 50 c.c. to 1 c.c. of sedimented bacteria, after thoroughly re-suspending the cells each time. The suspension was finally brought to opacity 2 on Brown's scale and a 2-mm. loopful inoculated into each tube. The platinum loop was ignited to red heat between two inoculations to destroy all adhering organic matter. The tubes were kept at 27°C. and observed at regular intervals for the appearance of turbidity due to growth. They were examined in the dark-room in the concentrated beam of a Pointolite lamp after wiping the outer surfaces of the tubes with a moist cotton to remove dust. This method enables the faintest appearance of growth to be detected.

Manometric experiments.—The stimulation of the respiration or the oxygen uptake of cultures of the organism was measured in Warburg manometers at 37°C. 2·00 or 3·00 c.c. of 2 to 7 days' old culture of the bacillus in nutrient broth were placed in the main chamber of each manometric vessel. The test compound in solution was placed in the side bulb and tipped into the culture at the commencement of measurement. Twenty per cent KOH was placed in the central well of the vessel on a roll of filter-paper to absorb the respiratory CO_2. For studying the effect of the compounds on the oxidations catalysed by bacterial suspensions, the organism was grown on agar in Roux bottles as usual but was collected and washed twice on the centrifuge in M/15 phosphate buffer at pH 6·5. Each manometric vessel contained bacterial suspension equivalent to 5 mg. (dry weight) bacteria in the side bulb and an excess of the substrate represented by 1·0 c.c. of M/10 solution in the main chamber. The quantities of the test compounds added to the vessels are given in the tables containing the results of these experiments. The oxidations were studied at 37°C. Further details regarding the manometric study of these oxidations are given in a previous communication (Rao, 1940). The following substrates were chosen for study : glucose, hexosediphosphate, pyruvate, lactate, and alanine. In some experiments proline and phenylalanine were also included. The selection of 37°C. for the study of metabolism instead of 27°C. previously adopted is based on experiments which show that the oxygen uptake of cultures is double at the former temperature than at the latter. The metabolism is also greater at pH 6·5 and at pH 7·5 than at the previously employed pH 7·0. However, collecting and centrifuging of the organism is preferable at pH 6·5 since a better sedimentation of the bacteria is obtained at this reaction.

EXPERIMENTAL RESULTS.

I. *The effect of the test compounds on growth in the amino-acid medium.*—The first series of experiments were on the activity of the six compounds, hæmatin,

cozymase, thiamin, nicotinic acid, β-alanine, and flavin, in reducing the lag period of growth of an inoculum in the amino-acid medium. The results are presented in Table II. The effect of each compound has been tested singly as well as in a few combinations with each other. The results show that hæmatin, of all the compounds tested, has the greatest effect on promoting the growth of the bacillus. Three other compounds : cozymase, thiamin, and nicotinic acid, have an identical but lesser activity. β-alanine has a slight effect. Flavin has no action whatever and is rather inhibitory in combination with other compounds. Hæmatin shows the maximum effect in combination with thiamin and nicotinic acid. The four compounds, hæmatin, cozymase, thiamin, and nicotinic acid, may therefore be considered to be growth stimulants.

TABLE II.

The effect of hæmatin and other compounds on the growth of the plague bacillus in an amino-acid medium.

| | | GROWTH IN :— | | |
		12 hours.	36 hours.	72 hours.
1.	Control	0	tr.	+
2.	„ + hæmatin (20γ/c.c.)	+	++	+++
3.	„ + cozymase (10γ/c.c.)	tr.	+	+
4.	„ + thiamin (10γ/c.c.)	tr.	+	+
5.	„ + nicotinic acid (20γ/c.c.)	tr.	+	+
6.	„ + β-alanine (20γ/c.c.)	tr.	tr.	+
7.	„ + flavin (0·02 c.c./c.c.)	0	tr.	+
8.	„ + hæmatin + thiamin	+	++	+++
9.	„ + hæmatin + thiamin + nicotinic acid ..	++	+++	+++
10.	„ + hæmatin + thiamin + nicotinic acid + β-alanine.	++	+++	+++
11.	„ + hæmatin + thiamin + nicotinic acid + β-alanine + flavin.	+	++	++
12.	„ + thiamin + nicotinic acid	tr.	+	++
13.	„ + thiamin + nicotinic acid + β-alanine ..	tr.	+	++
14.	„ + thiamin + nicotinic acid + β-alanine + flavin	0	0	tr.
15.	„ + nicotinic acid + β-alanine	tr.	+	+
16.	„ + nicotinic acid + β-alanine + flavin ..	0	0	tr.
17.	„ + β-alanine + flavin	0	0	tr.

1γ = 0·001 mg.   tr. = traces of growth.

II. *The effect of the growth stimulants on the respiration of growing cultures.*—If a compound has a stimulatory effect on the growth of the bacteria it may be expected to possess an activating effect on their metabolism as well. To test this possibility the effect of the above four growth stimulants on the O_2-uptakes of growing cultures of the bacillus were measured manometrically. Table III presents the results of a typical experiment. All the four compounds are found to increase the O_2-uptake of the culture. But the extent of the stimulation observed here does not strictly parallel the activity displayed in growth, the concentrations of the compounds to each other being kept the same for comparison. Nicotinic acid has now the greatest effect; next come thiamin, cozymase, and hæmatin in order. Hæmatin which has the greatest stimulation effect on growth has the least stimulation on respiration. Flavin, which was included for comparison, has an inhibitory effect.

TABLE III.

The effect of growth stimulants on the oxygen uptake of the plague culture.

(Two c.c. of a 72-hour-old culture per manometric vessel.)

		Oxygen uptake.
1. Broth-culture alone (control)	..	137 cu.mm. in 120 minutes.
2. „ + 100γ cozymase	..	152 „
3. „ + 200γ hæmatin	..	149 „
4. „ + 100γ thiamin	..	161 „
5. „ + 200γ nicotinic acid	..	169 „
6. „ + 0·2 c.c. flavin	..	126 „

III. *The effect of the growth stimulants on individual oxidations.*—The stimulation of growth and the stimulation of the O_2-uptake of cultures by the four compounds, hæmatin, cozymase, thiamin, and nicotinic acid, may be regarded as two different aspects of one and the same metabolic phenomenon. It is of much interest to know which of the oxidations of substrates that the bacillus is capable of carrying out are specifically influenced by these compounds. Out of the large number of oxidations that have been previously studied only a few of the more active ones have been selected for experimenting. Table IV presents the results of a manometric experiment on the effect of hæmatin on these oxidations. The

results show that hæmatin stimulates the O_2-uptake of the bacillus in the absence of substrates (the uptake in blanks). It has little effect on the oxidation of glucose, hexosediphosphate, lactate, alanine, and phenylalanine. It inhibits the oxidation of pyruvate and proline.

Similar experiments on the effect of cozymase on the oxidation of glucose, hexosediphosphate, pyruvate, and alanine, are given in Table V. The results show that cozymase stimulates the blank O_2-uptake as well as the oxidation of glucose and alanine. It is inhibitory on the oxidation of pyruvate. Cozymase does not stimulate the break-down of hexosediphosphate suggesting that this substrate may not be an intermediate in the oxidation of glucose.

TABLE IV.

The effect of hæmatin on some oxidations catalysed by the bacillus.

(200γ hæmatin per vessel.)

		Oxygen uptake.
1.	Control (no substrate)	57 cu.mm. in 60 minutes.
2.	Control + hæmatin	74 ,,
3.	Glucose	405 ,,
4.	Glucose + hæmatin	403 ,,
5.	Hexosediphosphate	144 ,,
6.	Hexosediphosphate + hæmatin	140 ,,
7.	Pyruvate	235 ,,
8.	Pyruvate + hæmatin	209 ,,
9.	Lactate	314 ,,
10.	Lactate + hæmatin	317 ,,
11.	Alanine	84 ,,
12.	Alanine + hæmatin	82 ,,
13.	Proline	102 ,,
14.	Proline + hæmatin	60 ,,
15.	Phenylalanine	68 ,,
16.	Phenylalanine + hæmatin	66 ,,

TABLE V.

The effect of cozymase on some oxidations catalysed by the bacillus.

(100γ crude cozymase per vessel.)

		Oxygen uptake.
1.	Control	33 cu.mm. in 60 minutes.
2.	Control + cozymase	59 ,,
3.	Glucose	236 ,,
4.	Glucose + cozymase	271 ,,
5.	Hexosediphosphate ..	175 ,,
6.	Hexosediphosphate + cozymase ..	165 ,,
7.	Pyruvate	181 ,,
8.	Pyruvate + cozymase	74 ,,
9.	Alanine	120 ,,
10.	Alanine + cozymase	130 ,,

Experiments on the effect of thiamin are reported in Table VI. Thiamin has considerable stimulatory effect on the oxidation of glucose; but it is inhibitory on the blank O_2-uptake. It has a marked inhibition on pyruvate oxidation. The latter observation is in contradiction to recent work on the catalytic rôle of this compound and its phosphoric ester in this reaction. A possibility which can be tested in future experiments is that, as shown by Barron and Lyman (*loc. cit.*), the plague bacillus may like *Gonococci* and *Streptococci* be unable to phosphorylate thiamin which *Staphylococcus* can do. Pyruvic acid is generally considered to be an intermediary product in glucose break-down. Since thiamin does not stimulate pyruvate oxidation in this organism but stimulates glucose oxidation it has evidently in the latter reaction a new catalytic rôle which remains to be elucidated.

In Table VII are presented the results of parallel experiments on the effect of nicotinic acid on the oxidations. Nicotinic acid is stimulatory on the oxidation

of glucose only ; it is inhibitory on the oxidation of pyruvate, lactate, and alanine, and on the blank O_2-uptake of the bacteria. Since the importance of nicotinic acid is assumed to be connected with the synthesis of the phosphopyridine nucleotides, a comparison between the effect of this compound and cozymase on the oxidations is interesting. Both stimulate glucose oxidation ; but while cozymase stimulates both alanine oxidation and the blank O_2-uptake, nicotinic acid does not stimulate either. Therefore, nicotinic acid probably does not give rise to cozymase in the cell. Whether triphosphopyridine nucleotide is produced can only be decided when the effect of the latter compound is tested. The above is the first instance in which nicotinic acid has been shown to activate a bacterial oxidation. It is possible that it has a similar effect on the metabolism of those bacteria for which it is known to be a growth factor.

TABLE VI.

The effect of thiamin on some oxidations catalysed by the bacillus.

(100γ thiamin per vessel.)

	Oxygen uptake.
Experiment 1.—	
1. Control	82 cu.mm. in 150 minutes.
2. Control + thiamin	63 „
3. Glucose	375 „
4. Glucose + thiamin	715 „
5. Hexosediphosphate	276 „
6. Hexosediphosphate + thiamin	266 „
Experiment 2.—	
7. Control	52 cu.mm. in 120 minutes.
8. Control + thiamin	25 „
9. Pyruvate	394 „
10. Pyruvate + thiamin	289 „

TABLE VII.

The effect of nicotinic acid on some oxidations catalysed by the bacillus.

(200γ nicotinic acid per vessel.)

		Oxygen uptake.	
1. Control	.. .. ..	27 cu.mm. in 60 minutes.	
2. Control + nicotinic acid	.. ..	19	,,
3. Glucose	.. .. ..	188	,,
4. Glucose + nicotinic acid	.. ..	224	,,
5. Hexosediphosphate	.. ..	82	,,
6. Hexosediphosphate + nicotinic acid	..	80	,,
7. Pyruvate	.. .. ..	129	,,
8. Pyruvate + nicotinic acid ..	..	110	,,
9. Lactate	.. .. ..	156	,,
10. Lactate + nicotinic acid	.. ..	146	,,
11. Alanine	.. .. ..	77	,,
12. Alanine + nicotinic acid	.. ..	55	,,

DISCUSSION.

The previous investigation of the nutritional requirements of the plague bacillus (Rao, 1939) has shown conclusively that growth factors of the complex type do not constitute compounds indispensable for the growth of the organism. The experiments described in the present paper on the other hand show that some of these compounds, in conformity to the general trend of recent work on the co-enzyme function of bacterial growth factors, stimulate the growth and the metabolism of the bacillus. Hæmatin to a remarkable extent, and cozymase, thiamin and nicotinic acid to a lesser extent, stimulate the growth of the bacillus in a chemically-defined medium. These four compounds also stimulate the respiration of the organism in actively-growing cultures. Finally, the latter three catalytically accelerate one or two of the individual oxidations of substrates effected by washed suspensions of the bacillus.

The fact that hæmatin is the most active compound hitherto found that reduces the lag in the growth of the plague bacillus has several interesting implications. Hæmatin is an iron-porphyrin compound with a pyrrole structure. The indispensability of the amino-acid proline, with its pyrrole nucleus, for growth is apparently connected with the synthesis of hæmatin or other porphyrin derivatives in the cell. Hæmatin is also the prosthetic group of hæmoglobin. It is significant,

therefore, that blood provides the best medium for the growth of the organism, particularly for the development of single isolated cells into colonies on agar. Such isolated cells are unable to develop on the ordinary nutrient-agar media. The stimulated growth of the bacillus on blood finds much use in the laboratory. Goré (1929a, b; *see* also Taylor, 1933) has devised routine methods based on the use of blood agar for the isolation of single-cell cultures and for the detection of contaminants in a pure culture of the organism. More recently, Sokhey (1939) has developed an exact technique of counts for the bacillus which is based on the counting of colonies on blood agar. An attempt to investigate the factors involved in the phenomenon was made by Schütze and Hassanein (1929) who showed that growth from scattered cells on agar is obtainable by using blood, sodium sulphite (*see* Drennan and Teague, 1917), by incubating in the absence of oxygen, or by adding a sterile filtrate from an old plague culture and from cultures of many other bacteria. They have put forward the theory, which has been supported by the work of Wright (1934), that the organism is sensitive to the presence of oxygen in the same manner as anærobes. They have further suggested that the active factor present in sterile filtrates is the enzyme catalase since (*a*) it is destroyed by heating to 70°C., (*b*) it is produced only by those bacteria which are known to be catalase producers, and (*c*) it can protect the bacteria against the most likely destructive factor in the presence of molecular oxygen which is hydrogen peroxide. From the present experiments it is clear that blood provides hæmatin itself, which has on the agar surface precisely the same effect on the growth of the bacillus as is observed in the synthetic medium. There is a close parallel between the ability of an isolated cell to grow into a colony on agar and that of the well-washed cells of the inoculum in the amino-acid medium overcoming the prolonged lag and multiplying. The necessity of hæmatin for the ærobic growth and multiplication of the plague bacillus is suggestive of the similar rôle of the compounds in *Hæmophilus*. It may be noted that hæmatin is the prosthetic group of catalase (Zeile and Hellström, 1930; Stern, 1936). The association of hæmatin with catalase, peroxidase, cytochrome, and other enzymatic systems connected with the reduction of molecular oxygen in cell respiration as in the nutrition of *Hæmophilus*, is also significant. Hypotheses such as these indicate the direction in which further progress can be made. The simplest way in which hæmatin can stimulate respiration is by combining with the nitrogenous compounds of the medium or of the cell to form hæmochromogens which can act as auto-oxidizable hydrogen carriers (Anson and Mirsky, 1925). This may occur in the manometric experiments with cultures since the curves indicating the stimulation of O_2-uptake (not presented above) do not display an induction period which must precede if hæmatin is to be assimilated and synthesized into more complex cellular constituents before it can act.

The importance of thiamin, cozymase, and nicotinic acid, as respiratory coenzymes in the cells of the bacillus may be the basis of their activity as growth stimulants. Experiments with the suspensions indicate that thiamin has a catalytic function in the oxidation of glucose which is unconnected with the break-down of pyruvate as an intermediary product. The stimulation of the same oxidation by

nicotinic acid is also a new and noteworthy fact. Considering that all the four growth stimulants increase the O. uptake of the cultures it is remarkable that no more than two oxidations are stimulated. This may be taken as indicating the existence of many other reactions in active growth of which we have little knowlelge and on which the specific effect of the compounds seems to be exercised.

The general significance of growth and metabolic stimulants in the life of the organism can be pointed out. It is more than probable that hæmatin, thiamin, cozymase, and nicotinic acid, occur in the cells of the bacillus as co-enzymes. Since the organism can grow in their absence it may be concluded that they are synthesized during growth. It may be suggested that the growth of the bacillus both *in vitro* and in the host is not conditioned by minimum indispensable requirements only but is greatly influenced by growth stimulants which probably in the main control the rapidity of growth and the extent of multiplication. The extraordinary invasiveness displayed by the bacillus finds a partial explanation in the fact that blood and hæmatin provide conditions greatly stimulatory to multiplication.

SUMMARY.

1. Experiments are described on the effect of hæmatin, cozymase (diphosphopyridine nucleotide), thiamin, nicotinic acid, alloxazine-adenine-dinucleotide. and β-alanine, on the growth and metabolism of the plague bacillus.

2. Hæmatin is highly active in reducing the lag in the growth of the bacillus in a chemically-defined amino-acid medium. Cozymase, thiamin, and nicotinic acid, possess similar but less marked activity. Hæmatin, thiamin, and nicotinic acid together, have the greatest effect recorded.

3. Hæmatin, cozymase, thiamin, and nicotinic acid, stimulate the respiration (oxygen uptake) of actively-growing cultures of the bacillus.

4. In a study of the effect of the compounds on the oxidation of a few of the more important substrates by washed suspensions of the organism it has been shown that hæmatin has no stimulatory effect on any of the oxidations studied, but increases the blank O_2-uptake of the suspension. Cozymase stimulates the oxidation of glucose and alanine and the blank O_2-uptake. Thiamin and nicotinic acid stimulate the oxidation of glucose only.

5. It is suggested that the four growth stimulants may be essential components of the cell, being synthesized in the course of growth, and that their occurrence and ready availability in the environment will greatly facilitate the rapidity of growth and of invasion of the organism.

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SANDFLY FEVER VIRUS: CERTAIN OF ITS PROPERTIES AND AN ATTEMPT AT PROPHYLACTIC VACCINATION.

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THE virus of sandfly fever, first isolated by the technique of cultivation on the chorio-allantoic membrane of the chick embryo (Shortt *et al.*, 1936) and later in tissue culture (Shortt *et al.*, 1938), has been maintained by serial passages through 72 and 66 generations, respectively. Various strains, derived from a variety of sources, have been maintained in the laboratory and have been used in the experiments described below.

PATHOGENICITY OF SANDFLY FEVER VIRUS TO LABORATORY ANIMALS.

Shortt *et al.* (1934) have shown that the virus as present in the blood of human cases of sandfly fever will produce modified but definite attacks of fever when inoculated into *Rhesus* monkeys. Shortt *et al.* (1938) obtained inconclusive results, as regards pathogenicity to mice, when the cultured virus was introduced by various routes. These observations were based on inoculations of virus where no special attempt to enhance the virulence of the strains had been made. In the present series of experiments it was hoped that serial passages of the strain at frequent intervals would increase its virulence for the animal under experimentation. White mice were used.

Intraperitoneal passages.—A mouse was inoculated intraperitoneally with 1 c.c. of a thirty-fifth passage flask culture. Three days later, about 1 c.c. of heart blood was taken into Tyrode solution and inoculated intraperitoneally into another mouse. The third day was chosen because we had previously ascertained that on this day virus was always present in the heart blood. In this manner seven serial passages were carried out in mice. The heart blood from the seventh serial passage mouse was inoculated intraperitoneally into six mice. Two of them were noted at the time to be somewhat sickly in appearance. These two mice died, one on the fourth and the other on the fifth day after inoculation, both without any characteristic symptoms. The remaining four mice remained healthy over an observation period of four weeks.

Intracerebral passages.—A mouse was inoculated intraperitoneally with 1 c.c. of tissue-culture virus. On the third day the mouse was killed and the brain passaged into a mouse intracerebrally. Subsequently seven serial passages were made. The mouse of the seventh passage experiment remained healthy for twelve days, although the presence of virus could still be demonstrated in the inoculum.

Observations on monkeys (M. sinicus).—The behaviour of the virus in monkeys (*M. sinicus*) was next studied. They were inoculated with cultured virus (tissue-culture virus) in large doses. Controls were inoculated with the tissue-culture medium without virus. The temperatures of all the monkeys were recorded for a period of two months. None of the monkeys showed significant temperature reactions nor any other response to the inoculations of virus. Details of this experiment are given in Table I :—

TABLE I.

Showing details of observations on monkeys inoculated with cultured sandfly fever virus.

Number of monkey.	Inoculum.	Dose in c.c.	Method of attempted infection.	Observation period.	RESULTS.
1 and 2	Normal embryonic tissue suspension with fowl plasma and normal human serum.	4·5	Subcutaneous	2 months	Monkeys remained apparently healthy.
3	20th passage tissue-culture virus.	5	do.	do.	Monkey remained apparently healthy.

TABLE I—*concld.*

Number of monkey.	Inoculum.	Dose in c.c.	Method of attempted infection.	Observation period.	RESULTS.
4	As in 3 plus a second inoculation of the 22nd passage tissue-culture virus 8 days after.	5 + 5	Subcutane-ous	2 months	Monkey remained apparently healthy.
5	25th passage tissue-culture virus plus second inoculation of 2Cth passage tissue-culture virus 12 days after.	5 + 5	do.	do.	do.
6	do.	5 + 5	do.	do.	do.
15	do.	5 + 5	do.	do.	do.
16	do.	5 + 5	do.	do.	do.
7, 8, 9, 10, 11, 12, 13, and 14.	25th passage tissue-culture virus.	5	do.	do.	Monkey 12 died on 21st day, of enteritis; probably an intercurrent infection.

PERSISTENCE OF SANDFLY FEVER VIRUS IN THE CIRCULATING BLOOD OF MONKEYS AFTER SUBCUTANEOUS INOCULATION.

The criterion used for proving the presence of virus in the circulating blood was the ability to grow it in culture on the chorio-allantoic membrane of chick embryos.

The sera of monkeys 3, 5, 6, 7, 8, and 10, used in the previous experiment, were tested for the presence of virus at varying intervals after the last inoculation.

Virus was demonstrated up to a maximum of 19 days in one monkey and was present in the majority up to 11 days. The details of the experiment are set forth in Table II :—

TABLE II.

Persistence of sandfly fever virus in blood of monkeys after its subcutaneous inoculation.

(5·0 c.c.)

Number of monkey.		Day of test after inoculation of virus.	Results of inoculation on chorio-allantoic membrane.*
3	{	8	+++, +, ++
		56	—, —, —, —
5		11	++, +++, +++
6		11	+, +++, +
7	{	11	} —, —, —, —
		19	
8	{	11	±, —, —, —
		19	±, —, —, —
10	{	11	+, +++, —, —
		19	++, —, —, —

*Note.—   +++
++
+   } = Degree of lesions produced in
±   individual eggs.
—

PRESENCE OF NEUTRALIZING ANTIBODIES IN THE SERA OF MONKEYS INOCULATED WITH SANDFLY FEVER VIRUS.

The technique adopted for demonstrating the presence of neutralizing antibodies was that described by Shortt *et al.* (1938) for the sera of convalescent human cases. It was found that antibodies could be·demonstrated up to at least 69 days

and its persistence beyond this period is being investigated. The details of this experiment are given in Table III :—

TABLE III.

Presence of neutralizing antibodies in monkeys inoculated with 5·0 c.c. cultured sandfly fever virus suspension.

Number of monkey.	Day of test after inocula-tion of virus.	Results of test on chorio-allantoic membrane.*
4	27	Serum alone : —, —, — Serum + virus : +, —, —, ±, — Virus alone : +++
5	64	Serum alone : —, — Serum + virus : —, —, —, — Virus control : +++, +, +, +, —
6	69	Serum + virus : —, —, —, ±, — Virus alone : +++, —, —
7	69	Serum + virus : ±, —, —, — Virus alone : +++
8	47	Serum + virus : —, —, —, — Virus alone : ++, +++, —, —

*Note.— +++
++
+
±
— = Degree of lesions produced in individual eggs.

PERIOD DURING WHICH VIRUS CIRCULATES IN THE BLOOD OF CASES OF SANDFLY FEVER AND OF CONVALESCENTS.

In this series of experiments blood was collected from patients on different days of the disease and also from convalescents. The results are set forth in Table IV and these are commented upon at the end of the table.

TABLE IV.

Showing results of inoculation of eggs with sera of sandfly fever cases taken at varying intervals after onset of the attacks of fever.

Day after onset of disease.	Number of sera tested.	Number of sera containing virus.	Number of eggs inoculated with positive sera showing		Maximum lesions obtained.*
			Lesions.	No lesions.	
1	1	..	..	..	—
2	13	4	8	15	+++
3	9	4	7	9	+++
4	8	4	8	7	+++
5	4	2	2	1 (4 putrid)	+++
6	7	4	8	9 (2 putrid)	+++
7	2	2	2	5	+ (focal lesions).
8	1	..	..	..	
21–30	8	4	7	12	++
31–40	6	2	5	8	++
41–50	3	1	1	3	Focal lesions.
51–60	4	..	..	..	—
61–70	6	1	1	2	+
71–80	2	1	1	1	±
81–90	1	1	3	2	+
91–100	4	..	..	..	— (3 putrid).
101–110	..	..	..	..	
111–120	4	..	..	..	—
121–130	..	..	..	..	—
131–140	1	..	..	..	—
141–150	1	..	..	..	—
151–160	3	1	1	2	±
161–170	1	..	..	..	—

*Note.— +++ ++ + ± — } = Degree of lesions produced in individual eggs.

All the sera tested, with the exception of nine, were dried and preserved. The results show that there is circulating virus in the blood in an appreciable number of cases up to three or four weeks after the first onset of fever. If a ++ lesion be taken as positive evidence of the presence of virus it is seen that circulating virus may be present up to 40 days after the onset of fever. The positive results for the 61 to 70, 81 to 90, and 151 to 160, groups must be accepted with reserve owing to the doubtful significance of the smaller and less distinct lesions. The fact that +++ lesions are only shown by the sera stored a short time appears to be evidence that aging of the sera is accompanied by a diminution of virus content. This, however, is not the entire explanation because all the nine sera which were not dried come among the earlier sera and it is also probable that the drying of the sera as well as their transportation across India may have reduced their potency as regards power of producing lesions.

It might be interesting here to note that there is evidence to suggest that the virus occasionally is present in the cerebro-spinal fluid also. Out of the four specimens of cerebro-spinal fluid tested by membrane culture technique, one produced passageable lesions on membranes. The cerebro-spinal fluid was taken, in this case, on the second day of the disease when the virus was also found to be present in blood. The identity of the virus was not, however, finally determined by cross neutralization tests and this finding will have to be confirmed by later work.

ATTEMPT AT PROPHYLACTIC VACCINATION OF HUMAN VOLUNTEERS WITH SANDFLY FEVER VIRUS VACCINE.

Composition of vaccine.—The technique of cultivating the virus in flask cultures has been described by Shortt *et al.* (1938) and it was from such cultures that the vaccine was prepared. A sufficiency of flask cultures being prepared, the contents were pooled, tested for bacteriological sterility and for virus content by culture by the chorio-allantoic membrane technique. The virus used was in its thirty-third passage in flask culture. The pooled material was stored in ampoules of 1-c.c. and 2-c.c. capacity and despatched in dry ice for trial on human volunteers in a non-endemic area. Needless to say, the vaccine was first tested on experimental animals and found to be innocuous.

Vaccine experiments.—In the conduct of these we are greatly indebted to Dr. R. O. A. Smith, I.M.D., of the Medical Research Department, for assistance in obtaining suitable volunteers and in the carrying out of the vaccination procedure.

Sixteen human volunteers from a non-endemic area were chosen for the experiments and divided into two groups of eight each. The members of one group were each given the vaccine subcutaneously in two doses of 1 c.c. and 1·75 c.c., respectively. Six of this group were given the second dose after a week's interval and two after 10 days' interval. The members of the second or control group were each given similar doses of normal saline solution at the same time as the vaccine was administered to the members of the first group. Volunteer No. 6 in the control group had had experimentally-induced sandfly fever four years previously by inoculation of infective serum.

Reactions following inoculations.—After the first dose of vaccine no reactions, either local or general, were noticed. After the second dose all complained of pain and tenderness at the site of inoculation on the day succeeding the inoculation. This local reaction disappeared in three to four days. No rise of temperature or other constitutional reactions were noted.

In the control group no reactions of any kind were noted.

ISOLATION OF SANDFLY FEVER VIRUS FROM VACCINATED VOLUNTEERS AFTER THE FIRST DOSE OF VACCINE.

As animal experiments had shown that the virus circulates in the blood for some days after subcutaneous inoculation it was natural to suppose that a similar

finding would be obtained in the case of human subjects. The blood of the vaccinated cases was taken on the fifth day after the first dose of vaccine and the serum tested for presence of virus as already described in the case of experimental animals. Volunteer No. 6, although in the control group, was also tested as he had had sandfly fever four years previously. The results of the tests showed that out of the eight vaccinated volunteers five gave definite evidence of the presence of circulating virus while in three the results were inconclusive, chiefly owing to the fact that the eggs used became putrid, an ever-present difficulty in using this technique in the hot season in a humid tropical climate. Control volunteer No. 6, as was expected, showed an absence of virus.

This experiment showed that the vaccine used must, at least, have contained live virus when the volunteers were vaccinated. The details are given in Table V :—

<div align="center">

TABLE V.

Isolation of sandfly fever virus from blood of volunteers inoculated with sandfly fever vaccine 1·0 c.c. (No. F. C. 33) on 17-6-1938; blood taken 5 days after, i.e., on 22-6-1938.

</div>

Number.	Name.	Results in eggs.*	REMARKS.
1	Tulsi ..	—, ±, P, ++, —, +++	....
2	Surtea ..	P, P, ++	....
5	Sadhu Ram ..	++, P, +, +	....
6	Passa ..	—, —, —, —	Not inoculated with vaccine, but was experimentally given S.F. in 1934.
7	Asa ..	—, ±, P, P	....
9	Cantoo ..	P, P, P	Result inconclusive.
12	Beig Lal ..	+++, ++, +++, ±	....
13	Devi Ram ..	—, P, P, —	Result inconclusive.
15	Sita Ram ..	+, P, —, +	....

*Note.—

$$\left.\begin{array}{c} +++ \\ ++ \\ + \\ ± \\ — \end{array}\right\} = \text{Degree of lesions produced in individual eggs.}$$

P. = Putrid.
S.F. = Sandfly.

THE PRESENCE OF NEUTRALIZING ANTIBODIES IN SERA OF THE VACCINATED
GROUP OF VOLUNTEERS AFTER ADMINISTRATION OF SANDFLY
FEVER VACCINE IN TWO DOSES.

The sera of seven out of the group of 8 of the vaccinated volunteers were tested
for the presence of neutralizing antibodies thirty-five days after administration
of the second dose of vaccine. The technique used in the animal experiments was
followed. The details are given in Table VI :—

TABLE VI.

*Presence of neutralizing antibodies in sera of vaccinated
volunteers, 35 days after the second dose of the
sandfly fever vaccine.*

Serum number.	Results.*
I ..	Serum alone : —, —
	Serum + virus : +, ±, —, —
	Virus alone : ++, +, —
II ..	Serum alone : P, P
	Serum + virus : —, —, —
	Virus alone : ++, +, —
V ..	Serum alone : P, +±, P, ±
	These lesions were due to contamination.
	Serum + virus : — —, ±. —
	Virus alone : ++, +. ± focal lesions.
IX ..	Serum alone : —. P, P, P
	Serum + virus : —, P, P, P
XII ..	Serum alone : —, —. —
	Serum + virus : —. ——
	Virus alone : P, P, P

*Note.— ++ ⎫
        + ⎬ = Degree of lesions produced in
        ± ⎭   individual eggs.
        —
P = Putrid.

TABLE VI—*concld.*

Serum number.	Results.*
XIII ..	Serum alone : —, —, — Serum + virus : +, + +, —, — Virus alone : + + +
XV ..	Serum alone : —, —, — Serum + virus : + +, + + Virus alone : + +, +

*Note.—
$$\left. \begin{array}{c} + + + \\ + + \\ + \\ - \end{array} \right\} = \text{Degree of lesions produced in individual eggs.}$$

It will be seen from the above table that there is some evidence of the presence of neutralizing antibodies in five of the sera, while antibodies are completely absent in sera numbers XIII and XV.

By comparing these results with those given in Table V it will be seen that sera which had originally shown the presence of circulating virus now showed the presence, in some degree, of neutralizing antibodies.

Of the two sera which failed to show the presence of antibodies, viz., XIII and XV, the former had previously shown an absence of circulating virus and the latter had contained them to a small degree only.

ATTEMPTS TO TRANSMIT SANDFLY FEVER TO VACCINATED AND CONTROL VOLUNTEERS BY INOCULATION OF INFECTIVE SERUM FROM CASES OF SANDFLY FEVER.

As stated previously, the sixteen volunteers were divided into two groups of eight each. These may hereafter be called 'vaccinated' and 'control' groups. When the test dose of infective serum was given, two volunteers out of the vaccinated and one out of the control group were not available for experiment. The remaining thirteen individuals were divided into four groups as follows :—

GROUP I.—Two vaccinated and one control volunteer.

GROUP II.—Two vaccinated and two control volunteers.

GROUP III.—One vaccinated and one control volunteer.

GROUP IV.—One vaccinated and three control volunteers.

All these individuals were kept under observation for a period of five days, pulse and temperature records being maintained. They were then given the infective dose of sandfly fever serum—1 c.c. per volunteer—subcutaneously, forty-two days after the second vaccination.

The infective serum used was not the same for each group. Twelve sera, proved to contain virus, were divided into four pools, each of which contained three sera and in each pool the three sera were mixed in equal volumes. Table VII gives details of the four pooled sera :—

<div align="center">

TABLE VII.

Showing results of inoculation of eggs with the sera used in the tests on volunteers.

</div>

Pool number.	Serum number.	Date of collection.	Results of test inoculation on chorio-allantoic membrane at the time of collection.*	Results of test inoculation on chorio-allantoic membrane of pooled sera at King Institute on receipt by post several days later.*
I	46/1	27–7–1938	+, +, ±	++, +, −
	44/1	27–7–1938	±, −, −	
	40/1	22–7–1938	+	
II	45/1	27–7–1938	++, ++	This pool was not tested at the King Institute, Guindy, as the ampoule was found to be contaminated.
	35/1	6–7–1938	++	
	31/1	1–6–1938	Not tested.	
III	43/1	26–7–1938	++, +	Inconclusive.
	42/1	26–7–1938	+	
	39/1	15–7–1938	+	
IV	41/1	22–7–1938	++	++, ++, ++, +, +
	36/1	11–7–1938	++	
	30/1	1–6–1938	Not tested.	

*Note.— ++ +, ±, − } = Degree of lesions produced in individual eggs.

Volunteer group I received pooled serum I, volunteer group II pooled serum II and so on. The volunteers in each group were kept under observation for a period of fourteen days after inoculation.

The details and results of inoculation are given in Table VIII :—

TABLE VIII.

Showing results of vaccination of volunteers—General summary.

Number and name.	Circulating virus after 1st dose vaccine.	Neutralizing antibodies after 2nd dose.	Infective human serum. Pool number.	Virus in infective serum.	Clinical symptoms after infective human serum inoculation.	Result of infective serum inoculation.	REMARKS.
12 Beig Lal vaccinated.	+++	+	I	+	Slight backache on the 2nd day and malaise on the 3rd day. Better on the 4th day. No other symptoms. T° normal.	No S.F. fever.	The infective serum pool used consisted of 3 sera, collected after 18, 20, and 30 hours after the onset of fever and injected into volunteers after 21, 10, and 10 days, respectively. Each serum had given + lesions in eggs in Peshawar; the pool serum tested at King Institute showed ++, +, –.
13 Devi Ram vaccinated.	*Nil?*	*Nil?*	I	+	No symptoms whatever.	No S.F. fever.	
16 Ganga Ram (control).	–	–	I	+	Local tenderness and ecchymosis at the site of inoculation. Slight T° (98·8) on the 10th day and malaise since injection. Temperature normal on the 11th day and pains in limbs. Does not look ill.	No S.F. fever. (Not likely that it was modified S.F. fever).	Results of the test are, therefore, inconclusive.
1 Tulsi vaccinated.	+++	+	II	+	8th day : had malaise, headache, myalgia, and photophobia with T° 100·4—pain in limbs. Appears a modified attack of S.F. fever. T° on one day only and improved subsequently. No malaria parasites in blood.	Modified S.F. fever.	The infective serum had virus

Name					Clinical notes	S.F. fever result	Remarks
5 Sadhu Ram vaccinated.	++	+	II	+	No symptoms whatever. No rise in T°.	No S.F. fever.	as tested in Peshawar. At King Institute serum found contaminated; so not tested. The serum proved infective. Apparently, the two vaccinated cases had some degree of protection and the attack in the control was really mild with T° of 100·4 for one day only. The other was fully protected (?).
3 Huri Ram (control).	—	—	II	+	History of malaria 12 months ago. Definite rigor on the 4th day. B. T. parasites in films (quinine not given). Normal next day (5th). 6th day patient said : felt feverish (?) but for 30 minutes only. 7th day : malaise. Severe frontal headache. 8th day : T° 99·0. No symptoms. 9th day : T° 101·0. General weakness, frontal headache, conjunctivæ suffused. No parasites. T° came to normal, 11th day and gradually improved.	S.F. fever (?) (See remarks).	The first control had malaria in the first instance. His second rise of T° might have been S.F. fever for the following reasons :— Clinical features, T° curve was of S.F. fever (and in contrast to the curve of B. T. infection) and no malaria parasites in blood, quinine having been withheld. 2nd control : a plainsman. Question is : had he any residual immunity due to artificially induced S.F. fever or other natural attacks of S.F. fever in plains ?
6 Passa (control).	—	—	II	+	Living on plains. Headache slight on the 5th day. On the 12th day T° 98·7 and cold in head. No complaints.	No S.F. fever.	

Note.— +++ ++ +± — } = Degree of lesions produced in individual eggs.

S.F. = Sandfly.

TABLE VIII—concld.

Number and name.	Circulating virus after 1st dose vaccine.	Neutralizing antibodies after 2nd dose.	Infective human serum. Pool number.	Virus in infective serum.	Clinical symptoms after infective human serum inoculation.	Result of infective serum inoculation.	Remarks.
15 Sita Ram vaccinated.	+	Absent	III	+	10th day: T° 99·8, malaise, frontal headache and myalgia, conjunctivæ suffused. 11th day: T° normal; but headache and conjunctiva suffused. 12th day: T° 99·6 with headache and backache. 13th day: T° normal and improved subsequently.	Modified S.F. fever. (Very mild).	The serum proved definitely infective. The vaccinated case had, if at all, a very mild attack with fever of a biphasic type. The control had a typical attack of S.F. fever. The vaccine appeared to have given at least a partial immunity. But it must be noted that circulating antibodies were not detected, but there was virus in the blood after 1st inoculation of vaccine.
14 Chaitu Ram (control).	—	—	III	+	6th day: headache; severe photophobia, T° 102·6, conjunctivæ very suffused. 7th day: T° 101·4, severe frontal headache, photophobia, myalgia. No malaria parasites in blood. 8th day: felt better; T° normal, but frontal headache and backache. 9th day: improved subsequently.	Typical S.F. fever.	

9 Cantoo vaccinated.	Inconclusive.	Inconclusive.	IV	+	6th day: T° 103·2, frontal headache, photophobia, and muscular pains. 7th day: T° 100·0, headache and myalgia, conjunctivæ suffused. No malaria parasites. 8th day: T° normal, improved subsequently.	Typical S.F. fever.
4 Shonnoo (control).	—	—	IV	+	4th day: malaise and feverishness (?). 6th day: feverish (?), frontal headache and myalgia. 7th day: better, conjunctivæ slightly injected. 8th day: improved. T° normal throughout.	No attack of S.F. fever.
10 Sukhan (control).	—	—	IV	+	On the third day feverish, but on the 10th day T° 99·6. No symptoms. Improved.	No S.F. fever.
11 Perida (control).	—	—	IV	+	No symptoms—T° normal.	No S.F. fever.

The infective serum on egg inoculation had shown definite evidence of virus both at Peshawar and at King Institute (++, ++, ++, +, +, +).

That it was infective is shown by typical attack in the vaccinated individual. Absence of reaction in the 3 controls is therefore not explained. Two were plainsmen. It must be noted that the vaccinated individual did not show definite evidence of either circulating virus or antibodies. Did he fail to react to vaccine? Results in this group are inconclusive as controls failed to react.

Note:— ++++ ++ ± = Degree of lesions produced in individual eggs.

S.F. = Sandfly.

DISCUSSION.

It must be admitted that the results of these preliminary attempts to produce a prophylactic vaccine against sandfly fever have been inconclusive. In the first place, the number of volunteers was too small. Out of eight originally selected for vaccination only six were available for the complete experiment. Of these, four showed the presence of circulating virus in the blood after the first dose of vaccine, while three of the four showed also the presence of neutralizing antibodies after the second dose of vaccine. Of these three, two escaped the infection altogether, while one showed a modified attack.

Of the remaining three, which either showed no virus after the first dose of vaccine or no neutralizing antibodies after the second, one had a typical attack of sandfly fever, one a modified attack and one escaped infection.

One might be tempted to assume some correlation between the presence of virus and circulating antibodies and the production of a degree of immunity but the numbers in the present experiment are totally inadequate to justify the drawing of any conclusions. Out of seven control volunteers who completed the experiment, two had typical attacks of sandfly fever and five escaped infection. The attacks were quite typical as compared with the modified attacks in the vaccinated group. The one typical attack in the latter might be accounted for by the absence of circulating virus and antibody.

If the results are analysed by groups, it is evident that the results in group I are inconclusive owing to the failure of the control to contract a typical infection.

In groups II and III there is some evidence of at least partial protection being conferred by the vaccine. In group IV the results are again inconclusive owing to the failure of the controls to contract infection.

We do not consider the comparative failure of the vaccine to give adequate protection as necessarily discouraging and believe that this purely preliminary work should be followed up and developed as further experience indicates. It is probable that the degree of protection conferred by virus vaccines, even live vaccines, may be, to some extent, dependent on dosage and it must be pointed out that the dosage used in the case of the human volunteers was very small, actually as well as relatively, when compared with the dosage of virus given by us to experimental animals. It is possible, therefore, that one method of increasing the efficacy of the vaccine would be to increase dosage. Another factor which has a bearing on the results is the degree of infectivity of the infective serum used to produce infection in the volunteers.

In previous experiments it was found (Shortt *et al.*, 1934, 1935) that out of twenty bloods from sandfly fever cases only eleven produced infection in volunteers. This indicates that there may have been considerable dilution of the infective virus in the serum pools used for infecting the volunteers and, from previous experience, it seems that a larger dose of infective serum may have to be used.

Both the factors dealt with above will have to be taken into consideration in repeating these experiments.

SUMMARY.

1. The virus of sandfly fever is in its 71st serial passage in eggs and its 62nd passage in flask cultures.

2. The cultured virus of sandfly fever was found to be non-pathogenic to mice and monkeys.

3. The virus could be recovered from the circulating blood of monkeys up to a maximum of 19 days.

4. In the sera of inoculated monkeys neutralizing antibodies could be demonstrated up to at least 69 days.

5. In cases of sandfly fever in human beings the virus was demonstrable in the circulating blood for seven days in most cases and up to 40 days or over in certain cases.

6. Sandfly fever vaccine containing live virus produced no marked general or local reactions in human volunteers.

7. The virus could be demonstrated in the circulating blood of vaccinated persons on the 5th day and neutralizing antibodies could be demonstrated up to 35 days after vaccination.

8. The results of vaccination in producing an effective immunity against infection with sandfly fever were inconclusive.

9. Certain factors to be considered in evaluating the results obtained are discussed.

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VITAMIN-D CONTENT OF THE LIVER AND BODY OILS OF BENGAL FISH.

BY

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ALTHOUGH the vitamin-A content of the body and liver oils of some varieties of fish in Bengal and other parts of India has been determined, no assay of their vitamin-D values appears to have been undertaken. This communication deals with the estimation of the vitamin-D content of the body and liver oils of some varieties of Bengal fish. Rachitic rats and chickens were used as experimental animals and the degree of healing was determined from the percentage of ash in the femur.

Halvorson and Palmer (1932) used chickens for the determination of vitamin D by the ash-content method, because of the fact that the vitamin-D requirement of the chick is high and correspondingly the results were trustworthy. The response of chickens in comparison with the response of rats to a given anti-rachitic agent, is the principal means of investigating the multiple nature of vitamin D (reviewed by Bills, 1935). Bills, Massengale and Imboden (1934) observed that the liver oil of one of the tuna species was less anti-rachitic than cod-liver oil, rat unit for rat unit, in chickens. The liver oils of twenty-five species of fish were assayed for vitamin D by Bills, Massengale and Hall (1937) in comparison with cod-liver oil on rats and chickens by the ash-content method. The maximum observed difference in relative effectiveness was about eighteen times.

EXPERIMENTAL.

Preparation of the oil.

Both the liver and body oils of fishes were extracted by the same process as was followed in the determination of vitamin A (Basu, Rai Sircar and Sen Gupta, 1940).

Technique.

A. Using rats as experimental animals.—Young albino rats about a month old (25 to 30 days) and weighing between 25 g. and 35 g. were obtained from a healthy and vigorous colony. The stock-breeding diet was kept as nearly uniform as possible throughout the year and was low in vitamin D.

The young rats were placed on a vitamin-D-free basal diet having the following composition :—

			Per cent
Ground yellow maize	..	..	76
Wheat gluten ..	..	..	20
Sodium chloride ..	..	..	1
Calcium carbonate ..	..	..	3
		TOTAL ..	100

The ratio of Ca : P was about 4 : 1. The animals given this diet were placed in cages shielded against sunlight with a black screen. The diet was given mixed with a little water to form a paste.

After four to five weeks on this diet, young rats showed signs of rickets which were detected by swollen wrists and other indications.

After the preparatory period, each of the animals was placed in a separate ·cage with a raised screen to prevent access to the excreta and shielded against light with a black screen as before. One rat of each litter was left on the basal diet only without any anti-rachitic supplements, thus serving as a negative control, while positive controls received a daily dose of a standard source of vitamin D, e.g., 0·2 units of international standard from a solution of an irradiated ergosterol preparation (League of Nations' standard). The other test animals were fed the basal diet plus a daily graded dose of the oils to be assayed, which was preferably fed as a supplement. The diluent used was olive oil and the oils were administered orally before the daily meal. Rats which lost weight or which failed to consume sufficient food were discarded.

The length of the test period necessary has been discussed by various workers. In the present investigation the test period lasted from 9 to 14 days and was counted from the first day of dosing. The aim of the experiment was to find a dose of the test substance which, given daily for the test period to young rats on the above rachitogenic diet, would cause the production of bones having the same percentage of ash as those produced when some selected daily dose of international standard was administered.

After the end of the test period the animals were killed by coal gas, the bones of both hind legs were carefully dissected free from all flesh (the patella being also removed), washed in boiling water for a minute and then dried at 100°C. for 24 hours. The dried bones, wrapped in silk, were extracted first with boiling alcohol for about 16 hours, and then with ether in a Soxhlet apparatus for 24 hours. After extraction the bones were placed in a weighing bottle, dried to constant weight,

and removed to a weighed platinum crucible to be ashed. The ash was heated to constant weight (Chick, Korenchevsky and Roscoe, 1926).

B. *Using chickens as experimental animals.*—A colony of fowls was maintained on a normal ration containing, at all seasons, about 200 international units of cod-liver oil vitamin D per 100 g. of feed. Eggs from these hens were selected for hatching. All eggs with an imperfect shell or an abnormal shape and all which weighed less than 55 g. the day they were laid, were discarded. No food was given to the chickens before the third day of life. From the third until the seventeenth day of life all chickens were fed on the following rickets-producing diet M and given tap-water to drink :—

Diet M.

	Parts.
Ground yellow maize	56
Wheat bran	10
Linseed oil meal	10
Wheat gluten	10
Skimmed milk powder	9
Calcium carbonate	2
Sodium chloride	1
Vegetable oil	2

$$Ca : P = 2 : 1$$

After two weeks on the diet, diseased chicks, weaklings, and runts, either died or were thrown out. The remaining strong chicks almost without exception survived the subsequent test periods.

The best length of the test periods has been discussed by several workers. According to Massengale, Charles and Bills a period of four weeks is the most suitable. In the present investigation the test period lasted for two to three weeks and was counted from the first day of dosing.

At the end of the fore-period on the seventeenth day of life, the chicks were transferred to assay cages. Five to ten chicks were placed in each cage. In such close quarters the birds were restricted as to exercise which might or might not be a factor of importance. The cages were shielded against sunlight with a black screen. Chickens constituting the standard test group were given diet M plus the supplement of vitamin D in doses of 0·4 international units daily, thus serving as a positive control. Some animals were kept on the diet M only during the test period, thus serving as a negative control. The best and most accurate results are obtained by a comparison of the response of the group of animals obtaining a given dose of oil with the response of another group obtaining a dose of some standard of reference, the two tests being carried out simultaneously. The remaining animals were then given in addition to the basal diet graded doses of oil daily as supplement. The diluent used was olive oil and the supplement was administered orally before the daily meal.

At the end of the test period the chickens were killed. One femur from each bird was immediately dissected out, and placed in boiling water for one minute. It

was then scraped free of adherent tissue, care being taken to avoid removal of the still cartilaginous epiphyses (Bethke and Record, 1934). The bones were then broken and extracted in a Soxhlet apparatus for 12 hours with alcohol and 12 hours with ether. The extracted bones were dried to constant weight at 100°C., weighed, then ashed to constant weight in an electric muffle furnace at 700 ± 20°.

RESULTS.

A. With rats.—Table I gives the average ash content of bones of rats given various doses of oils, and Table II shows the vitamin-D values of the different oils.

B. With chickens.—In Table III will be found the average ash content of bones of chickens given various doses of oils, and Table IV shows the vitamin contents of the different oils.

TABLE I.

The average ash content of the femoral bones of rats given various doses of oils.

Name of the supplement.	Zoological name of the fish.	Number of rats used.	Dose in mg.	Days under experiment.	Average percentage of ash.
Nil	..	6	..	9	53·8
Standard vitamin-D solution	..	6	0·2 unit	9	55·3
Ruhee-liver oil ..	*Labeo rohita*	5	10	9	57·56
Kātal-liver oil ..	*Catla catla*	7	10	9	54·12
Mrigel-liver oil ..	*Cirrhina mrigala*	6	10	9	54·49
Kói-body oil	*Anabus testudinus*	5	20	9	52·00
Nil	..	6	..	9	53·88
Standard vitamin-D solution	..	7	0·2 unit	9	55·00
Naindal-liver oil ..	..	5	50	9	52·91
Hil-á-body oil ..	*Clupea ilisa*	7	50	9	60·36
Air-liver oil	*Arius arius*	7	50	9	56·32

TABLE II.

The vitamin-D values of different oils measured by rat experiments.

Name of the oil.		Zoological name of the fish.	Units of vitamin D per g.	Weight of oil (g.) containing one unit.
Ruhee-liver oil	..	*Labeo rohita*	51·83	0·0192
Hilsā-body oil	..	*Clupea ilisa*	23·14	0·0432
Mrigel-liver oil	..	*Cirrhina mrigala*	8·59	0·1164
Air-liver oil ..	..	*Arius arius*	7·57	0·1321
Kātal-liver oil	..	*Catla catla*	3·38	0·2958
Naindal-liver oil	..	..	*Nil*	..
Kôi-body oil ..	..	*Anabus testudinus*	*Nil*	..

TABLE III.

The average ash content of the femoral bones of chickens given various doses of oils.

Name of the supplement.		Zoological name of the fish.	Number of chicks used.	Dose in mg.	Days under experiment.	Average ash percentage.
Nil ..	..	..	7	..	22	36·37
Ruhee-liver oil	..	*Labeo rohita*	7	20	22	65·75
Standard vitamin-D solution		..	7	0·4 unit	22	42·70
Kātal-liver oil	..	*Catla catla*	7	20	22	36·53
Air-liver oil ..	..	*Arius arius*	6	20	22	36·97
Sarputi-liver oil	..	*Barbus sarana*	7	20	22	36·00
Māgur-body oil	..	..	6	50	22	39·65
Nil ..	..	..	6	..	14	35·13
Standard vitamin-D solution		..	7	0·4 unit	14	40·51
Naindal-liver oil	..	..	7	50	14	36·53
Hilsā-body oil	..	*Clupea ilisa*	6	50	14	35·81
Chital-body oil	..	*Notopterus chitala*	7	50	14	48·95

TABLE IV.

*The vitamin-D values of different oils measured by experiments
with chicks.*

Name of the oil.	Zoological name of the fish.	Units of vitamin D per g. of the oil.	Weight of the oil (g.) containing one unit.
Ruhee-liver oil ..	*Labeo rohita*	92·82	0·0107
Chital-body oil ..	*Notopterus chitala*	20·55	0·0486
Māgur-body oil ..	..	4·14	0·2415
Naindal-liver oil ..	..	1·93	0·5181
Air-liver oil	*Arius arius*	1·89	0·5291
Hilsā-liver oil	*Clupea ilisa*	1·01	0·9900
Kātal-liver oil ..	*Catla catla*	0·52	1·9230
Sarputi-liver oil ..	*Barbus sarana*	*Nil*	..

DISCUSSION.

The relative effectiveness of the different oils is not identical in experiments with rats and chickens. This is in agreement with the observations of Bills *et al.* (1934, 1937) and indicates the multiple nature of vitamin D. Ruhee (*Labeo rohita*) liver oil appears to be the most potent source of vitamin D and is almost twice as effective in curing rickets in chickens as in rats. Hilsā-body oil, which is fairly potent in rats, shows very little anti-rachitic activity in chicken. It may be remembered that Ruhee-liver oil is a good source of vitamin A, containing 461 international units per g., while Hilsā-body oil is devoid of vitamin A (Basu and De, 1938). Naindal-liver oil which is a very good source of vitamin A (Basu, Rai Sircar and Sen Gupta, *loc. cit.*) shows very little anti-rachitic potency towards chicken and none towards rats. Although not a very good source of vitamin D, Air-liver oil is about four times more effective in rats. Chital-body oil appears to be fairly potent in chickens.

It would appear that compared with the anti-rachitic potency of the cod-liver oil which contains 150 to 200 rat units per c.c., the vitamin-D content of liver

oils of Bengal fish is small. The most potent oil, the Ruhee-liver oil, is only about one-third as active as cod-liver oil.

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NUTRITIONAL INVESTIGATIONS ON BENGAL FISH.

BY

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IN a previous communication (Saha and Guha, 1939) we have given the analysis of 24 different varieties of Bengal fresh-water fish for water, body fat, ash, protein, calcium, phosphorus, total iron, and ionizable iron. The results of analyses of 13 more varieties of Bengal fish are given in the present communication. As certain varieties of fish are not available throughout the year, it is necessary to carry on the investigation from season to season.

EXPERIMENTAL.

The methods employed in these investigations are the same as described in the previous paper. The mean values given in Tables I and II are based on analyses on two or more days with four to seven samples of each variety of fish. Dates of analyses are given in order to indicate the season. The zoological names of a few of these have not been obtained.

TABLE I.

Mean values of water, body fat, ash, and protein per 100 g. of fish.

Date of collection.	Bengali name.	Zoological name.	Ranges of body-weights (g.).	Water (g.).	Body fat (g.).	Protein (g.).	Ash (g.).	Average percentage accounted for.
19–1–39 21–1–39	Rohu Source—Jheel	*Labeo rohita*	8650—168	77·1	2·2	15·2	1·3	95·8

TABLE I—*concld.*

Date of collection.	Bengali name.	Zoological name.	Ranges of body-weights (g.).	Water (g.).	Body fat (g.).	Protein (g.).	Ash (g.).	Average percentage accounted for.
23–1–39 22–1–39 2–2–39	Bāchā ..	*Chpisoma garua*	178—75	68·8	5·6	18·1	1·4	93·9
6–2–39 8–2–39	Bām ..	..	291—45	74·8	0·9	16·1	1·3	93·1
28–2–39 1–3–39	Bhole ..	*Sciaena coitor*	590—78	78·1	1·1	15·2	1·9	96·3
3–3–39 6–3–39	Kharsālā ..	..	180—67	75·3	5·1	16·3	1·8	98·5
17–1–39 18–1–39 15–1–39	Dhāin ..	*Silonia silundia*	9534—417	72·0	12·1	14·0	1·4	99·1
16–1–39 19–1–39	Kātlā .. Source—Jheel	Catla catla	9732—523	74·2	1·9	18·6	1·6	96·3
21–1–39 22–1–39 19–1–39	Sarputi ..	*Barbus sarana*	381—107	70·2	9·5	16·5	1·53	.97·7
14–2–39 16–2–39	Pābdā ..	..	100–35	73·0	2·1	19·2	1·14	95·4
25–1–39 27–1–39 29–1–39	Pangas ..	*Pangasius pangasius*	3650—632	72·3	10·8	14·2	0·96	98·3
18–2–39 21–2–39	Roynā ..	..	119—48	76·0	1·1	15·6	1·20	93 9
10–2–39 12–2–39 3–2–39	Pākal ..	..	45—20	76·8	1·2	14·3	1·08	93·4
2–2–39 3–2–39	Air ..	*Arius arius*	3020—1032	78·1	1·3	15·9	1·15	96·b

TABLE II.

Mean values of calcium, phosphorus, total iron, and ionizable iron per 100 g. of fish.

Bengali name.	Zoological name.	Calcium (g.).	Phosphorus (g.).	Total iron (mg.).	Ionizable iron (mg.).
Rôhu ..	*Labeo rohita*	0·62	0·20	1·08	0·61
Bāchā ..	*Chpisoma garua*	0·52	0·18	0·68	0·43
Bām ..	..	0·33	0·24	0·84	0·37
Bhole ..	*Sciacna coitor*	0·55	0·58	0·42	0·20
Kharsālā ..	..	0·41	0·16	0·64	0·42
Dhāin ..	*Silonia silundia*	0·36	0·24	1·04	0·58
Kātlā ..	*Catla catla*	0·55	0·26	0·94	0·49
Sarputi ..	*Barbus sarana*	0·22	0·12	0·54	0·32
Pābdā ..	..	0·31	0·21	1·32	0·43
Pangas ..	*Pangasius pangasius*	0·18	0·13	0·52	0·51
Roynā ..	..	0·12	0·08	0·36	0·04
Pākal ..	..	0·13	0·11	0·42	0·10
Air ..	*Arius arius*	0·38	0·18	0·66	0·26

SUMMARY.

Tables I and II give the mean values for water, body fat, total mineral matter, protein, calcium, phosphorus, total iron, and ionizable iron, in 13 different varieties of fish, obtained usually from analyses of four to seven samples of each kind. Among the fish analysed Dhāin (*Silonia silundia*) has the highest fat content, 12·1 per cent, then come Pangas (*Pangasius pangasius*), 10·8 per cent, and Sarputi

(*Barbus sarana*) 9·48 per cent. The protein content, 19·2 per cent, is highest in Pābdā. The value of calcium, 0·62 per cent, is highest in Rôhu (*Labeo rohita*). The next best sources of calcium are Kātlā (*Catla catla*) and Bāchā (*Chpisoma garua*), which contain 0·55 per cent and 0·52 per cent of calcium, respectively. The phosphorus content, 0·58 g., is highest in Bhole (*Sciacna coitor*). As regards ionizable iron, Rôhu is the best source containing 0·61 mg. per cent. The question of iron in fish is discussed in the next paper (Saha and Guha, 1940).

ACKNOWLEDGMENT.

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* *Erratum.*—Saha and Guha (1939): Read one-hundredth of the values for 'Total iron' in Table II as corrected values.

AVAILABLE IRON IN FISH.

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THE availability of iron in foodstuffs has been a subject of considerable discussion. An earlier belief was that it was the organic iron (Bunge, 1885) which was nutritionally useful. But recent researches (Elvehjem *et al.*, 1933; Lintzel, 1931; Whipple and Robscheit-Robbins, 1927) have shown that a considerable proportion of the organic iron present in foodstuffs is in the form of hæmatin-like compounds and is not absorbed by the system. Of late the ionizable iron content of foodstuffs has been considered to be nutritionally available and this has been estimated by the a-a'-dipyridyl method of Hill (1930). This method involves the use of a suitable reducing agent which reduces the ferric iron to the ferrous state. This iron and the naturally occurring ferrous iron, which ionize in solution, react with Hill's reagent, while the hæmatin iron does not. The total ionizable iron is thus estimated.

On a critical examination of the results obtained by the dipyridyl method, compared with those obtained by biological assay, it appears (McCance, 1939) that the iron not determined by a-a'-dipyridyl cannot be entirely accounted for as hæmoglobin or hæmatin, and the nature of the fraction not accounted for has not yet been ascertained. According to the work of Tompsett (1935) with human tissue, some of the iron appears to form complexes with phospho-proteins present in the tissue. He found that this complex is formed by the ferric and not by the ferrous iron and the complex is unstable in presence of sodium hydrosulphite which is used in Hill's method for the reduction of ferric iron. If this is so, then Hill's method would estimate the total ionizable iron including the iron of the iron-phospho-protein complex. We have found in our experiments that whether sodium hydrosulphite is added to a mixture of fish tissue and acetate buffer (of pH 5·5) or it is added to the extract of fish tissue, made with buffer, the value of iron obtained on subsequent treatment with Hill's reagent is the same.

We considered it of interest to investigate the action of proteolytic enzymes (pepsin and trypsin) on fish tissue in order to throw light on the question. If an iron-protein complex is present in the tissue, which would not normally react with a-a'-dipyridyl, the iron might react after proteolysis and subsequent reduction by hydrosulphite. If this is the case, then the iron of the iron-protein complex, even if the complex is insoluble in water, would also be available for nutritional purposes and Hill's method for estimating the available iron would need to be modified by first carrying out a proteolysis of the animal tissue concerned followed by reduction with sodium hydrosulphite and treatment with dipyridyl.

The method adopted was as follows : The edible portion of fish was separated from the bones and other non-edible portions. Ordinarily the fleshy portion with its small proportion of inseparable fatty tissue was taken. The large-sized piece thus obtained was first cut into thin slices, which were then thoroughly disintegrated in an ' Enterprise ' hasher. The mass was then ground into a pulp in a glass mortar. Ten grammes of the sample were weighed, treated with 20 c.c. of distilled water, and heated for 5 minutes as in Hill's method. In the case of pepsin digestion the pH of the solution was adjusted to 2·2 with hydrochloric acid, and in the case of trypsin digestion the pH was adjusted to 8·0 with 0·1N Na_2CO_3 solution and a few drops of toluene were also added in the latter case. Ten c.c. of 0·4 per cent solutions of Merk's pepsin and trypsin were added in different flasks which were incubated at 37°C. for 4 hours. The pepsin and trypsin digests were subsequently brought to pH 5·5 with the addition of Na_2CO_3 and hydrochloric acid respectively, when 10 c.c. of acetate buffer (pH 5·5) and 0·5 g. of (iron-free) sodium hydrosulphite were added in each flask. After standing overnight the mixture was centrifuged, the clear liquid decanted off and in each flask 2 c.c. of a-a'-dipyridyl (0·2 per cent) were added. After 24 hours, the colour developed was matched against the standard.

In order to compare the value of the iron obtained by this method with that obtained by the method of Hill, iron in the same sample was also determined by the simple a-a'-dipyridyl method described before (Saha and Guha, 1939). The results are given in Table I. In a few cases the zoological names of the fish were not obtainable.

TABLE I.

Available iron (in mg. per 100 g.) in fresh raw fish, as estimated by the a-a'-dipyridyl method before and after enzymic hydrolysis.

Bengali name.	Zoological name.	Weight of the fish in g.	After pepsin digestion.	After trypsin digestion.	Hill's method.
Mrigal ..	*Cirrhina mrigala*	163	0·52	0·50	0·43
		279	0·58	0·55	0·46
		534	0·69	0·66	0·45

TABLE I—*contd.*

Bengali name.	Zoological name.	Weight of the fish in g.	After pepsin digestion.	After trypsin digestion.	Hill's method.
Hilsã	*Clupea ilisa*	531	0·72	0·68	0·58
		732	0·83	0·81	0·62
		854	0·82	0·79	0·57
Shôle	*Ophcephalus striatus*	412	0·73	0·63	0·53
		731	0·80	0·69	0·52
		927	0·85	0·70	0·56
Bélé	*Glassgobius giuris*	25	0·11	0·08	0·06
		83	0·16	0·13	0·03
		206	0·15	0·12	0·05
Shinghi	*Saccobranchus fossilis*	16	1·42	0·82	1·10
		19	1·36	0·69	1·20
		32	1·48	0·91	0·83
		56	1·39	0·88	0·96
Bhetkã	*Lates calcifer*	256	0·62	0·52	0·32
		632	0·58	0·55	0·40
Kôi	*Anabas testudineus*	25	1·21	0·73	0·65
		36	1·36	0 68	0·66
		48	1·12	0·86	0·73
Pãrsey	*Mugil parsia*	6	0·73	0·71	0·48
		17	0·81	0·67	0·51
Bãgdã chingri	....	5	0·96	0·76	0·72
		9	0·88	0·81	0·67
		26	1·10	0·82	0·63

TABLE I—*concld.*

Bengali name.	Zoological name.	Weight of the fish in g.	After pepsin digestion.	After trypsin digestion.	Hill's method.
Air	*Arius arius*	233	0·53	0·48	0·32
		616	0·62	0·52	0·39
Mowrālā	*Amblypharyn godon-mola.*	3	0·62	0·48	0·42
		5	0·58	0·61	0·40
Kharsālā	....	26	0·66	0·43	0·41
		48	0·62	0·51	0·37
Pangas	*Pangasius pangasius*	1123	0·71	0·63	0·52
		1738	0·76	0·55	0·56
Kātlā	*Catla catla*	432	0·76	0·63	0·48
		678	0·72	0·56	0·46
		1132	0·75	0·67	0·53
Bôal	*Wallago attu*	572	0·59	0·52	0·41
		811	0·63	0·61	0·39
Māgur	*Clarius batrachus*	63	1·30	0·63	0·69
		76	1·10	0·72	0·73
		198	1·20	0·76	0·72
Rôhu	*Labeo rohita*	206	0·83	0·62	0·52
		352	0·76	0·53	0·58
		873	0·78	0·66	0·57

The above results indicate that the iron values given by most samples of fish tissue are much greater when estimated after peptic or tryptic digestion. A few samples of Shinghi, Māgur, and Rôhu, however, give by Hill's method values higher than those obtained after tryptic digestion, but in all cases without exception peptic digestion liberates the largest amount of iron. The reason why digestion by pepsin liberates more iron than tryptic digestion may be that the acid medium in peptic

digestion helps in the liberation of iron (*vide infra*). As the fish tissue would undergo normal proteolytic break-down in the system, the above results show definitely that application of Hill's method without previous enzymatic hydrolysis is likely to give low results for nutritionally available iron.

   The rate of liberation of iron from the iron-protein complex of fish tissue.—In order to investigate further the question of the iron-protein complex in fish tissue, the rate of liberation of iron was also studied by varying the period of digestion by pepsin, as this enzyme gives the maximum iron values. The results obtained with four different varieties of fish are shown in the Graph :—

GRAPH.

Period of peptic digestion in minutes.

Legend:

△ —— *Saccobranchus fossilis.*
○ — *Cirrhina mrigala.*
○ —— *Clupea ilisa.*
✕ —— *Lates calcifer.*
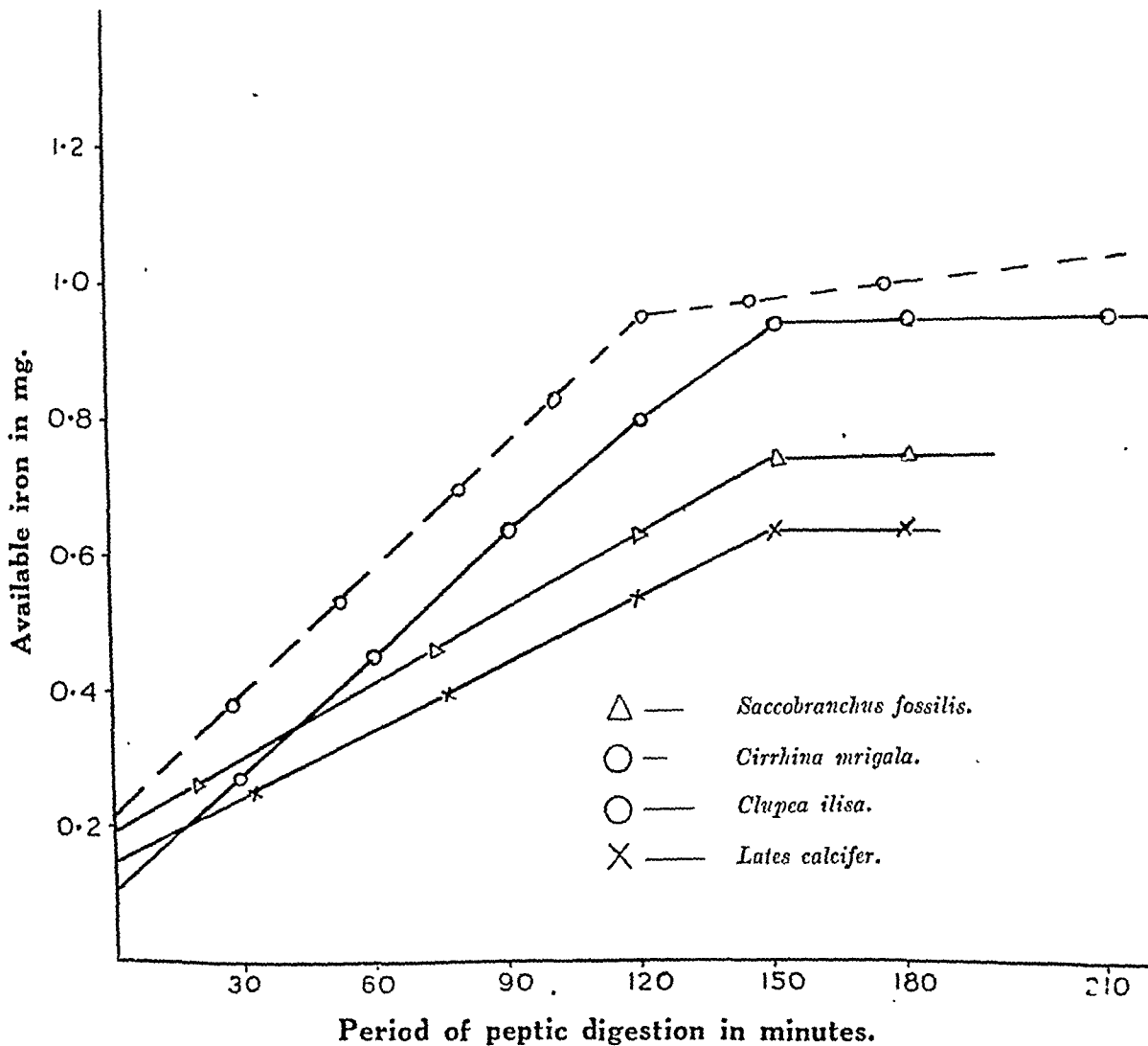
   The Graph indicates that the available iron value increases with the period of digestion and within 2 to 3 hours the liberation of iron appears to be complete.

This would indicate further that *in vivo* the iron of this iron-protein complex is completely available.

Extraction of fish tissue with buffer.—In order to investigate whether the iron-protein complex is extractable with buffer, a sample of fish tissue was estimated for total available iron by the method described above, viz., by a-a'-dipyridyl method after peptic digestion and reduction by sodium hydrosulphite. Another sample was treated as follows: 10 g. of the sample were treated with acetate buffer (pH 5·5) and with 0·5 g. of sodium hydrosulphite and the mixture was left overnight. Next day, the mixture was centrifuged. The residue was washed three times with distilled water in order to make it free from hydrosulphite and then digested with pepsin solution and its iron content estimated by a-a'-dipyridyl as described before. The iron content of the buffer extract was estimated by Hill's method without proteolysis. The values are given in Table II :—

TABLE II.

Available iron in mg. per 100 g. of fresh raw fish.

Bengali name.	Zoological name.	After peptic digestion of the fresh tissue.	By Hill's method applied to the buffer extracts and washings.	After peptic digestion of the fish residue.	Total available iron in buffer extract and residue.
Kôi ..	*Anabas testudineus*	1·23	0·76	0·45	1·21
Shinghi ..	*Saccobranchus fossilis*	1·38	0·95	0·40	1·35
Māgur ..	*Clarius batrachus*	1·26	0·71	0·42	1·13
Rôhu ..	*Labeo rohita*	0·85	0·50	0·32	0·82
Kātlā ..	*Catla catla*	0·72	0·49	0·21	0·70
Bāgdā chingri	....	0·93	0·76	0·15	0·91

From the above results it appears that practically all the iron-protein complex of the fish remains in the residue after extraction with the buffer, so that the value for total available iron obtained by peptic digestion of the raw fish is roughly equal to the amount of ionizable iron in the fish extract plus the amount of iron liberated after peptic digestion of the residue.

Available iron in fish eggs.—Certain varieties of fish obtained from the same sources and having approximately the same weights with and without eggs have

been investigated. It has been found that in the fish with eggs, the available iron is greatly diminished in muscle and is concentrated in the roe. This is comparable with what occurs in human beings, in whom during pregnancy the fœtus gets rich in iron (Sherman, 1937). The results are given in Tables III and IV. These also show the difference in available iron both in muscle tissue and in eggs by Hill's method and by our method :—

TABLE III.

Available iron in mg. per 100 g. of egg and muscle tissue of egg-bearing fish.

Bengali name.	Zoological name.	Weight of the fish in g.	Iron in muscle after peptic digestion.	Iron in muscle by Hill's method.	Iron in egg after peptic digestion.	Iron in egg by Hill's method.
Hilsã	*Clupea ilisa*	612	0·36	0·24	0·96	0·73
Bélé	*Glassgobius giuris*	113	0·18	0·07	0·48	0·33
Shinghi	*Saccobranchus fossilis*	46	0·63	0·50	1·85	0·98
Kôi	*Anabas testudineus*	52	0·53	0·36	1·63	1·32
Rôhu	*Labeo rohita*	415	0·36	0·25	0·97	0·72
Kãtlã	*Catla catla*	517	0·41	0·32	1·10	0·81

TABLE IV.

Available iron in mg. per 100 g. of the muscle tissue of non-egg-bearing fish.

Bengali name.	Zoological name.	Weight of the fish in g.	Iron in muscle after peptic digestion.	Iron in muscle by Hill's method.	Iron in egg after peptic digestion.	Iron in egg by Hill's method.
Hilsã	*Clupea ilisa*	587	0·76	0·51	..	..
Bélé	*Glassgobius giuris*	128	0·15	0·04	..	..
Shinghi	*Saccobranchus fossilis*	51	1·32	0·89	..	..
Kôi	*Anabas testudineus*	48	1·10	0·68	..	..
Rôhu	*Labeo rohita*	401	0·72	0·52	..	..

The effect of extracting fish tissues at different pH.—It was found that in certain tissues, only a small portion of the iron present reacted with dipyridyl when suspended in the sodium acetate-acetic acid buffer at pH 5·5 as recommended by Hill.

Investigations of Sherman (1934) showed that the buffering systems of these tissues were so strong that their action predominated over that of the added buffer. The result was that the pH of the tissue and buffer mixture did not remain at 5·5, but rapidly returned to a nearly neutral reaction which prevented the liberation of that portion of iron which was in simple chemical combination. The effect of increasing the acidity of the fish-tissue suspension on the value of available iron was therefore studied. All other conditions were maintained as in the method of Hill. The results are shown in Table V. For purposes of comparison the results of similar experiments carried out with goat meat (muscle tissue) are shown in the same table. These indicate that fish and meat behave similarly in this respect and in every case there is increased liberation of available iron as the acidity increases.

TABLE V.

Available iron in mg. per 100 g. of raw fish.

Bengali name.	Zoological name.	With buffer at pH 5·5.	WITH ACETIC ACID OF VARIOUS CONCENTRATIONS.					
			2 per cent.	4 per cent.	6 per cent.	8 per cent.	10 per cent.	12 per cent.
Bôal	*Wallago attu*	0·32	0·41	0·45	0·46	0·53	0·53	0·52
Bélé	*Glassgobius giuris*	0·03	0·07	0·12	0·13	0·14	0·13	0·13
Kātlā	*Catla catla*	0·52	0·61	0·76	0·85	0·84	0·85	0·85
Shinghi	*Saccobranchus fossilis*	0·87	0·93	1·20	1·25	1·30	1·30	1·31
Kôi	*Anabas testudineus*	0·76	0·86	0·88	0·96	0·97	0·97	..
Latā	*Ophicephalus punctatus*	0·65	0·72	0·76	0·76	0·76	0·75	0·76
Goat's muscle	....	0·23	0·46	0·65	0·88	0·86	1·45	..
,,	....	0·12	0·38	0·59	0·82	0·93	1·12	1·12
,,	....	0·30	0·46	0·63	0·91	1·10	1·30	1·30

From the above findings it is clear that simple extraction with buffer at pH 5·5 by Hill's method would not extract all the available iron. The lowest pH

values at which the maximum quantity of available iron is liberated vary from tissue to tissue. If, as in our method, the tissue is digested with pepsin prior to the buffer extraction then the disturbance caused by the buffering action of the tissue is eliminated and the iron values obtained are independent of the acidity of the medium which is shown in Table VI. This holds good also for goat-muscle tissue (Table VI) :—

TABLE VI.

Tissue digested with pepsin, prior to the addition of buffer or acetic acid at various concentrations. Figures are given in mg. of available iron per 100 g. of raw tissue.

Bengali name.	Zoological name.	With buffer pH 5·5.	WITH ACETIC ACID.			
			2 per cent.	4 per cent.	6 per cent.	8 per cent.
Shinghi ..	*Saccobranchus fossilis*	1·30	1·25	1·30	1·31	..
Rôhu ..	*Labeo rohita*	0·82	0·82	0·81	0·82	0·81
Bôal ..	*Wallago attu*	0·51	0·50	0·51	0·52	0·51
Bélé ..	*Glassgobius giuris*	0·13	0·13	0·13	0·13	0·12
Kātlā ..	*Catla catla*	0·85	0·84	0·84	0·84	..
Goat's muscle	....	1·35	1·38	1·36	1·35	1·38
,, ..	....	1·10	1·11	1·12	1·10	..

SUMMARY.

1. Some of the iron present in fish tissue appears to be present as an iron-protein complex which, as such, does not react with a-a'-dipyridyl. This iron-protein complex can be easily hydrolyzed by pepsin and trypsin and is therefore nutritionally available. After digestion the iron liberated from the iron-protein complex can be estimated by the a-a'-dipyridyl method of Hill. A method of estimating the total available iron is described, which involves peptic digestion followed by reduction with sodium hydrosulphite and treatment with a-a'-dipyridyl. The available iron estimated by this technique is much greater than that obtained with the undigested tissue by Hill's method. This also applies to animal tissues like goat's muscle. Treatment with 10 per cent acetic acid also gives values, which are practically equal to those obtained after peptic digestion, so that application of Hill's method after previous treatment with 10 per cent acetic acid would also appear to be a good alternative method for the estimation of available iron. In this method only a slight uncertainty is involved, as the minimum acid concentration

for getting the maximum value for available iron varies from fish to fish. With the varieties of fish investigated, however, 10 per cent acid concentration appeared to be sufficient for getting the maximum yield of available iron.

2. The peptic digestion of fish tissue gives considerably higher values than tryptic digestion. Peptic digestion is complete within two to three hours so far as available iron is concerned.

3. During egg-formation, the available iron content of the muscle of the fish is greatly decreased, while iron is found concentrated in the roe.

ACKNOWLEDGMENT.

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DIETARY AND NUTRITION SURVEY AT JAMSHEDPUR, AN INDUSTRIAL TOWN IN BIHAR.

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INTRODUCTION.

A FRUIT of modern industrial enterprise, Jamshedpur (popularly known as Tatanagar) has risen on the Chotanagpur plateau within the last thirty years, and at present ranks as one of the biggest industrial centres in the whole of India, and covers an area of about 25 (24·57) square miles. · The town is irregularly rhomboid in appearance, situated between the Subarnarekha and Kharkai rivers at the north-eastern portion of the Singhbhum district. The last (1931) census enumerated a population of 83,738 in the town but even by modest estimate the mid-year population 1938 comes to a figure slightly in excess of one lakh. As the town has grown almost out of nothing, or at best a few huts of the aboriginal tribes, the residents are all immigrants-from different parts of India. The town is well laid out with piped water-supply and sewerage system. The indices for the vital happenings at Jamshedpur compiled from the Annual Reports of the Medical Officer of Health for three successive years and enumerated in Table I, show unusually favourable rates as compared to the other parts of this province. In spite of the fact that Messrs. Tata Iron & Steel Company (with their subsidiary industries in the neighbourhood) employ people *with 'better than average' state of physique in their*

TABLE I.

Vital indices of Jamshedpur.

Individual index.	1936–37.*	1937–38.*	1938–39.*
Birth rate ..	8·85	10·49	10·4
Death rate ..	3·69	3·99	4·3
Infant mortality ..	45	30	28

* Year commences 1st April.

active periods of life, still this selected age and sex composition of the population cannot fully explain away such lowering of natality and mortality rates. Moreover, the registration of births and deaths is not compulsory in the town. All these facts tend to prove that some amount of under reporting in the Vital Registers did exist during the period under review.

An investigation into the dietary habits and state of nutrition of the residents was started by the author in the month of July 1938 extending over a period of little more than three months. Altogether 177 family units consisting of 845 persons were included in the diet survey and 2,003 children examined so as to discover their state of nutrition.

The dietary investigations were carried out on the lines suggested by Aykroyd and Krishnan (1937). Each family was under observation for fifteen consecutive days. The raw food before cooking was weighed and converted into proximate principles of food (protein, fat, etc.) for purposes of calculation according to the values laid down in Health Bulletin No. 23 (1938) and a publication by the author (Mitra, 1938) on the subject. The international scale of family co-efficients advocated by the Health Organization of the League of Nations (1932) was used in calculating intake per consumption unit or man-value. The energy values of the nutritive principles in the dietary were calculated according to Rubner's co-efficients.

An attempt has been made in Table II to divide the families surveyed in different income groups, altogether four in number. For references in the text the group numbers would be referred to for the sake of brevity. At Jamshedpur the minimum wages paid to the employees being eight annas and nine pies *per capita per diem.* In families with more than one earning member all the earnings have been pooled together in calculating the income of the family.

TABLE II.

Distribution of families surveyed into various economic groups.

Classification of families.	Total daily income of the families in rupees.	Number of families in the group.	Total number of con- sumers.	Total consump- tion units (man- values).	Average number of consumers per family.	Daily income per consumption unit (man-value) in the group, in annas (16 annas = 1 rupee).
Group I ..	Up to 1 ..	110	479	368·0	4·4	3·1
,, II ..	,, 1·8 ..	35	177	137·7	5·1	5·4
,, III ..	,, 3 ..	19	116	84·7	6·1	7·4
,, IV ..	Over 3 ..	13	73	62·7	5·6	14·0
	TOTALS ..	177	845	653·1	4·8	5·2

As the time at the disposal of the author did not permit of a comprehensive survey of all the different sects, communities, and classes, of the immigrants from all over India, the investigation was primarily limited to the natives of the province of Bihar. Out of a total of 177 families as many as 131 or 74 per cent belonged to this province. The frequency distribution of the families in each group investigated according to their native provinces is given in Table III. The families were picked up at random from amongst the residents of Sonari, Burma Mines, Kashidih, Sakchi, Sitaramdera, Bhalubasa, East Plant, Mahaulbera, and Dhatkidih *mahallas* of the town. Quite a large percentage of the householders in the *mahallas* surveyed looked at this operation with a certain amount of suspicion at the beginning, nevertheless the majority did not object to the weighment of food-stuff nor withhold any information sought for. Those protesting too much were either not taken in or else dropped out after a day or two. The investigations were confined to the families of the working classes, i.e., those earning their liveli-hood by manual labour. The majority of the bread-winners were employees of Messrs. Tata Iron & Steel Company and the rest were employed by the subsi-diary industrial concerns in the locality. No other special methods of sampling were employed in choosing the families.

TABLE III.

Frequency distribution of the provincial origin of the families surveyed in Jamshedpur.

Province.	Group I.	Group II.	Group III.	Group IV.	TOTALS.
Bihar	74	29	15	13	131
Central Provinces, including states.	15	5	..	..	20
Orissa, including states ..	20	1	3	..	24
Bengal	1	..	..	..	1
United Provinces ..	..	..	1	..	1

In the assessment of the physiological value of the diets adequacy of energy value or gross quantitative rating deserves primary consideration. Scales of calorie requirements for different occupations in India have yet to be worked out. Consequently one has to fall back upon recommendations made by the Technical Commission of the League of Nations (1938). The Commission was of opinion that an allowance of 2,400 calories daily was adequate 'for a sedentary man' and 3,000 calories for a moderately active man. Within the last few years some

amount of work on the basal metabolism rate has been carried on in different parts of India and the B. M. R. has been found to be definitely below the Aub and DuBois standards. Recently at Bombay, Niyogi *et al.* (1939), in working out energy expenditure budget, found that a man engaged in 'moderate work' requires 2,604 calories daily. Under the circumstances it would not be far out to classify the allowances in families with (*a*) intake of less than 2,600 calories *per capita* per diem as 'below standard', (*b*) with 2,601 to 3,000 calories as 'bare minimum', and (*c*) with an allowance of 3,001 calories and above as 'adequate'.

The frequency distribution of the families according to the total calorie consumption in the light of the arbitrary classification suggested above in each of the income groups is shown in Table IV. From Table IV it is quite apparent that almost all the families with inadequate calories in their diet belong to group I. The individual families in groups II and III judged 'below the standard' included nine and eleven persons, the calorie consumption being 2,440 and 2,517, respectively.

TABLE IV.

Frequency distribution of the families in the different income groups according to the adequacy of calorie consumption.

Calorie consumption.	GROUP I.		GROUP II.		GROUP III.		GROUP IV.	
	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.
'Below standard', up to 2,600 ..	31	28·2	1	2·8	1	5·3	*Nil*	..
'Bare minimum', 2,601–3,000 ..	22	20·0	8	22·9	6	31·6	2	15·4
'Adequate', over 3,000 ..	57	51·8	26	74·3	12	63·1	11	84·6
TOTALS ..	110	100·0	35	100·0	19	100·0	13	100·0

TYPES OF FOOD CONSUMED.

Before considering the proximate principles of food consumed daily by the individuals in the different groups of families, it is desirable to tabulate the respective intake in ounces in terms of raw food classified according to their nutritional

characteristics. Table V gives the average daily consumption in ounces of the various types of edibles in the different groups. The author (Mitra, 1939) has demonstrated that such a tabulation serves as a rough and ready index in the absence of a more detailed study.

TABLE V.

The types of food consumed daily in ounces per man-value in the different groups.

Families in different income groups.	Cereals.	Pulses.	Non-leafy vegetables.	Green leafy vegetables.	Oils and fats.	Flesh foods.	Milk and milk products*.	Fruits and nuts.	Condiments.	Sugar and jaggery.
Group I ..	23·9	2·4	2·3	1·2	0·5	0·6	0·5	0·1	0·7	0·2
„ II ..	24·4	3·1	2·7	1·0	0·8	0·7	1·4	0·3	1·0	0·3
„ III ..	22·1	3·8	5·5	0·3	1·3	1·3	2·6	0·9	1·6	0·7
„ IV ..	21·0	3·4	6·2	0·1	1·8	1·0	5·7	0·9	1·6	0·8
AVERAGE ..	22·9	3·2	4·2	0·7	1·1	0·9	2·6	0·6	1·2	0·5

* Does not include ' ghee '.

Almost all the families under investigation used one of the commoner vegetable oils expressed from mustard seeds (*Brassica juncea* and other species), gingelly seeds (*Sesamum indicum*), or mahua seeds (*Bassia latifolia*). Those who could afford to spend, supplemented the different vegetable oils with certain quantities of ' ghee ' (butter fat). Table VI gives the frequency distribution of families according to the intake of the different types of fats in the dietary. The table is self-explanatory and needs very little comment. Quite in keeping with the experience of other investigators the consumption of fats and oils was found to maintain a positive correlation with the increase of income in the groups (*see* Tables V and VI). On a detailed analysis it was further found that of the total fats used ' ghee ' comprised by weight 19·0 per cent, 40·5 per cent, 50·1 per cent, and 55·7 per cent, respectively, in groups I to IV.

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TABLE VI.

Frequency distribution of families according to the intake of different types of culinary fats.

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Income groups.		Total number of families.	FAMILIES USING THE FOLLOWING FATS.				
			Number of oil or 'ghee'.	Mustard oil only.	Gingelly oil only.	Mahua oil only.	'Ghee' plus any oil.
Group I {	Actual	110	2	76	11	1	20
	Percentage	100·0	1·8	69·1	10·0	0·9	18·2
„ II {	Actual	35	0	12	2	0	21
	Percentage	100·0	..	34·3	5·7	..	60·0
„ III {	Actual	19	0	2	0	0	·17
	Percentage	100·0	..	10·5	..	..	89·5
„ IV {	Actual	13	0	1	0	0	12
	Percentage	100·0	..	7·7	..	..	92·3
All families {	Actual	177	2	91	13	1	70
	Percentage	100·0	11·2	51·4	7·3	0·6	39·5

Coming to the consideration of protective foods, one finds average consumption increasing with the income with the exception of green leafy vegetables which shows a reverse correlation. But if the intake of non-leafy vegetables and green leafy vegetables be added up together then the higher income groups do show an increased rate of consumption. When the prices of the more common edibles at Jamshedpur are discussed later in the text it will be seen that green leafy vegetables are no dearer than the non-leafy ones. Why do the people in the higher income groups consume less of the former than their circumstances apparently permit? It is probably due to their ignorance of the nutritive values of the edible green leaves. During the survey operations the author noticed edible green creepers on the top of some of the thatched houses inhabited by the poorer class of people. This healthy practice accounts for the comparatively higher average consumption of 'shag' (green leaves) in the poorer income groups. Wilson and Mitra (1938), in a dietary survey with 37 families of industrial workers in Assam, found that each person per day was consuming 19 oz. cereals, 1 oz. pulses, 3·4 oz.

non-leafy vegetables, 0·2 oz. leafy vegetables, 0·3 oz. fats and oils, 0·2 oz. flesh foods, 0·5 oz. milk, and no fruits. This average daily intake compares unfavourably even with the lowest income group (group I) of Jamshedpur workers. The Assam workers were also comparatively poorly paid, their wages being Rs. 7 to Rs. 12 per month. Table VII gives the actual number as also the percentage of the families not consuming the different protective foods. This table has been introduced because the deviations round the arithmetical mean or average figures in all the groups shown in Table V does not give a correct picture of the happenings. Further, Table VII helps to give a picture of the trend of the dietary habits in the different income groups as far as the protective foods are concerned.

TABLE VII.

Families in the different income groups not consuming various protective foods.

Family groups.		Green leafy vegetables.	Flesh foods.	Milk.	Fruits and nuts.
Group I	Actual	33	45	79	95
	Percentage	30·0	40·9	71·8	86·4
„ II	Actual	12	9	14	28
	Percentage	34·3	25·7	40·0	80·0
„ III	Actual	9	4	3	10
	Percentage	47·4	21·1	15·8	52·6
„ IV	Actual	9	5	1	3
	Percentage	69·2	38·5	7·7	23·1

COST OF EDIBLES.

It is well known that the cost of the various commoner foodstuffs at Jamshedpur is much higher than the prices prevalent in other parts of this province. Of the cereal foods parboiled rice formed the staple article of diet. It was gratifying to find that the poorer classes invariably consumed home-pounded parboiled rice available in *hāts* (village markets). This type of coarse rice was sold at 26 lb. to the rupee. The costliest rice consumed in higher income groups was found to sell at 20 lb. to the rupee. Some amount of wheat flour (one anna per lb.) and green maize was consumed. Of the pulses red gram (arhar) was most popular. Some amount of

Bengal gram, green gram, and lentil, was consumed. The pulses used were sold at 12 lb. to 14 lb. per rupee. The common vegetables consumed were potato, parwar, raddish, bitter gourd, marrow (nenua), lady's fingers (bhindi), these were selling at two-and-a-half annas, four annas, three annas, three annas, two annas, and two-and-half annas, per pound respectively. Mustard oil and gingelly were selling at three annas to four annas per pound and 'ghee' at twelve annas per pound. The flesh foods consisted of goat meat (four annas per lb.), fish (six annas per lb.), fowl (four annas per lb.), and eggs (ten annas to the score). A certain amount of beef (two annas and six pies a lb.) was consumed by the Mohammedans and aboriginal families surveyed. Green leafy vegetables were selling at one anna and milk at two annas per pound.

PROXIMATE PRINCIPLES OF FOOD.

Table VIII gives the average consumption *per capita* per diem of the various proximate principles of food in families belonging to the different income groups. The corresponding figures calculated for the industrial workers in Assam as calculated by Wilson and Mitra (*loc. cit.*) are 58·8 g. protein of which 2·7 per cent being of animal origin; 11·99 g. of fat of which 3·2 being of animal origin; 458 g. of carbohydrate, 0·173 g. of calcium, 1·67 g. of phosphorus, 1,697 international units of vitamin A, 624 international units of vitamin B_1, and 26 mg. of vitamin C. The average intake of calories by Assam workers is 2,181 of which 88·9 per cent was contributed from cereal foods. If vitamin A be left out of consideration then the diet of the working-class families at Jamshedpur is decidedly superior both in quality and quantity as compared to that of similar families surveyed in Assam.

Protein.—The intake of total protein may be considered adequate, but that of the animal protein is certainly below standard in all the groups. With the increase of income level the average consumption figure for animal protein does show an increase.

Fat.—According to the minimum intake level advocated (45 g. to 60 g.) in the Health Bulletin (*loc. cit.*) the consumption of this item of food is below the level, except in the two higher income groups. The consumption of total fat and animal fat ('ghee') maintains a positive correlation with the income.

Calcium.—According to the Sherman (1937) standard (0·68 g.) the intake was inadequate in all the income groups. This is not at all surprising in view of the sparing consumption of milk and leafy vegetables (*see* Table V) and the high intake of rice.

Phosphorus.—Intake can be called adequate by the Sherman standard (1·32 g.) but with the increase of knowledge about phytin phosphorus one does not feel happy about the figures. The Ca : P ratio varies from 1 : 3 to 1 : 6 in extreme cases.

The intake of the other important mineral element, iron, has been excluded from the calculations as the figures for the availability of this element in all the foods consumed during the survey could not be found in the literature.

TABLE VIII.

Average daily intake of nutrients per man-value in the different groups.

Family groups.	Protein (g.).	Percentage of animal protein.	Fat (g.).	Percentage of animal fat.	Carbohydrate (g.).	Calcium (g.).	Phosphorus (g.).	Vitamin A (inter-national units).	Vitamin B₁ (inter-national units).	Vitamin C (mg.).	Total calories.	Percentage of calories from cereals.	Percentage of calories from fat.
Group I ..	68·4	4·3	21·1	9·5	605	0·41	1·39	2,013	861	70	2,941	83·9	4·0
,, II ..	78·2	7·1	35·2	27·1	623	0·51	1·58	1,981	1,015	69	3,191	74·8	7·0
,, III ..	84·8	8·7	45·2	36·9	607	0·55	1·66	1,833	961	68	3,247	68·0	9·2
,, IV ..	85·1	11·6	68·4	47·1	571	0·62	1·70	1,505	913	44	3,329	61·8	14·7
AVERAGE ..	79·1	7·9	42·5	30·2	601·5	0·52	1·58	1,833	938	63	3,177	72·1	8·7

Vitamin A.—The intake of this extremely important food principle is much below the universally-accepted standard in all the income groups. On a detailed analysis of the figures one finds that the percentage of families showing adequate consumption (3,000 international units *per capita* per diem) of this vitamin in groups I to IV are 19·1, 17·2, 21·1, and 23·1, respectively.

Vitamin B₁.—The level of intake of this vitamin was found to be higher than the prescribed minimum of 300 international units daily. Such high figures are not unusual in a population consuming daily about 20 oz. of parboiled rice and requisite amount of pulses. It may be added in passing that beri-beri is a disease rarely encountered in this province.

Vitamin C.—The minimum standard laid down in case of this vitamin is 30 mg. *per capita* per diem. From Table VIII it appears that the average intake of this vitamin is adequate in all the income groups. But on a closer study intake level of the percentage of families coming up to the standard from groups I to IV are found to be 60·0, 80·0, 78·9, and 84·6, respectively.

DISCUSSIONS ON IMPROVEMENT.

Out of a total of 177 families surveyed the calorie consumption of 33 families appears to be inadequate. In other words, a little over 18 per cent of the families were not getting enough to eat. This defect in the dietary can only be remedied if more money be spent on food either by increasing the income of the family to a certain extent or else by re-adjustment of the family budget if this be possible. The problem can only be solved by an experienced economist. The amount of indebtedness and expenditure on major items other than food could not be investigated as a detailed economic survey was beyond the scope of the author's investigations and the circumstances did not permit of it.

Regarding the qualitative defects in the dietary, these can be classified under (a) defects which can be remedied with very little or no cost and (b) defects which necessitate a certain amount of expenditure. In the former group can be included inadequacy of calcium, vitamin A, and vitamin C (in a small percentage of cases).

A rich source of vitamins A and C as also of calcium is the leafy vegetable. It is admitted that green edible leaves are comparatively dear at Jamshedpur, but every one of these householders can with very little expense grow the common creepers of gourd, marrow, pumpkin, etc., and use the green leaves. Commoner type of amaranth can also be grown. In this connection all that is necessary is the dissemination of the proper knowledge or propaganda, amongst the householders.

Increase of animal protein in the diet would certainly involve some amount of extra money in the case of the lower income groups. In the higher income groups, i.e., those with an income of Re. 1-8 or more, can certainly provide for this important food factor by increasing the consumption of milk or flesh foods, preferably the former.

EXAMINATION OF CHILDREN.

Altogether 2,003 children between the ages 2 and 16 years were examined anthropometrically as also clinically. This number consisted of 1,610 boys and 393 girls. These were picked at random in the neighbourhood of the *mahallas* surveyed and also from the primary schools. During the survey an attempt was made to confine the investigation to the state of nutrition of children up to 12 years age but where older children spontaneously or at the instigation of parents and guardians volunteered themselves for examination it was found very difficult to refuse them. The height and weight measures of the boys and girls in the different age groups are given in Table IX. Comparison with corresponding figures from the

TABLE IX.

Height and weight of boys and girls in the different age groups.

Age in years (last birth-day).	TOTAL NUMBER EXAMINED.		HEIGHT IN INCHES.		WEIGHT IN POUNDS.	
	Boys.	Girls.	Boys.	Girls.	Boys.	Girls.
2	9	14	33·00	31·64	21·1	18·0
3	22	16	35·45	35·00	26·4	24·0
4	41	31	41·49	39·10	29·7	27·8
5	144	63	42·96	42·36	34·2	32·2
6	241	82	45·04	44·39	38·5	34·0
7	241	55	47·51	46·47	44·2	40·8
8	229	49	50·02	48·79	48·6	45·9
9	267	32	51·37	50·15	52·2	49·3
10	183	28	53·18	52·78	57·3	53·4
11	156	20	54·64	53·20	63·4	56·1
12	39	1	56·33	54·08	65·0	62·0
13	18	2	57·36	55·0	76·3	70·5
14	10	..	58·92	..	85·3	..
15	7	..	60·71	..	89·2	..
16	3	..	61·67	..	91·7	..
TOTALS ..	1,610	393	..	..	..	..

different parts of India has been avoided as the children belonged to more than one race. The proportion of boys and girls in Table IX is in no way comparable to the existing distribution of the sexes amongst the children population at Jamshedpur. As boys could be secured comparatively easily during field work, their number predominated. The income of the children's parents or guardians could not be obtained, consequently they have been grouped under one class; only a distinction of sex has been made.

STATE OF NUTRITION.

All these children were rated as 'good', 'fair', and 'poor', as to their state of physique and general appearance by the naked-eye examination. This is the way of rating followed by the Medical Officers of the Public Health Department in the examination of the school·children of this province. The results are given in Table X :—

TABLE X.

Naked-eye assessment of the physique of children.

Sex.	Total number examined.	Good.		Fair.		Poor.	
		Number.	Percentage.	Number.	Percentage.	Number.	Percentage.
Boys ..	1,610	218	13·5	1,144	71·1	248	15·4
Girls ..	393	71	18·1	291	74·0	31	7·9
TOTALS ..	2,003	289	14·4	1,435	71·6	279	13·9

When the child looked definitely malnourished he was classified as 'poor' and when the child looked healthy, bright, and cheerful, irrespective of his robustness, he was classified as 'good'. The lustre of the eyes, the prominence of the ribs, and the skin conditions, were particularly noticed in arriving at a diagnosis. Others were all classified as 'fair'.

INCIDENCE OF DEFICIENCY CONDITIONS.

Each one of these children was examined for the physical signs of xerophthalmia, phrynoderma, angular stomatitis, dental caries, and malocclusion of teeth,

conditions supposed to be associated with one or other forms of dietary deficiency. The teeth figures for the dental caries represent the results of naked-eye examination as to dental decay. Of all the conditions noted above, the incidence of dental caries seems to be the highest, though cases of dental decay as noticed by the naked eye have only been recorded. Wilson and Mitra (*loc. cit.*) have recorded a much lower caries rate amongst the children of the comparatively poorly paid Assam industrial workers. The incidence of xerophthalmia is, however, much lower than the respective figure for Assam children records. 'Clinical signs' in Table XIII means only xerophthalmia, phrynoderma, and angular stomatitis.

TABLE XI.

Incidence of various conditions said to be associated with dietary deficiency.

Sex.	Number examined.	CHILDREN FOUND AFFECTED WITH									
		XEROPH-THALMIA.		PHRYNODERMA.		ANGULAR STOMATITIS.		DENTAL CARIES.		MALOCCLU-SION.	
		Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.
Boys ..	1,610	105	6·5	27	1·7	56	3·5	471	29·3	260	16·1
Girls ..	393	20	5·0	0	..	7	1·8	125	31·8	51	13·0
TOTALS ..	2,003	125	6·2	27	1·3	63	3·1	596	29·8	311	15·5

ARM-CHEST-HIP MEASUREMENT.

The A. C. H. index of almost all the children (1,579 out of 1,610 boys and 356 out of 393 girls examined clinically) was recorded during the survey operations. Those left out belonged to the extreme age groups (*see* Table IX). The percentage of children selected was as high as those of Assam labour population recorded by

Wilson and Mitra (*loc. cit.*). Tables XII and XIII give the results of analysis of the records :—

TABLE XII.

Four-fold table showing the number of children examined for clinical signs and A. C. H. index.

		Children with clinical signs.	Children not with clinical signs.	TOTALS.
Children selected by A. C. H. index.	Boys ..	64	512	576
	Girls ..	12	81	93
	All children ..	76	593	669
Children not selected by A. C. H. index.	Boys ..	52	951	1,003
	Girls ..	10	253	263
	All children ..	62	1,204	1,266
All children examined.	Boys ..	116	1,463	1,579
	Girls ..	22	334	356
	All children ..	138	1,797	1,935

TABLE XIII.

The comparative study of A. C. H. index and incidence of clinical signs.

Sex.	Number examined.	Percentage selected by A. C. H. index.	Percentage with clinical signs.	Percentage of those with clinical signs selected.	Percentage of those without clinical signs selected.
Boys ..	1,579	36·5	7·3	55·2	35·0
Girls ..	356	26·1	6·2	54·5	24·3
TOTALS ..	1,935	34·6	7·1	55·1	30·6

A glance at Table XIII suggests that the girls displayed a better state of nutrition than the boys. But statistical analysis of the figures shows that as far as the incidence of clinical signs is concerned the difference in rate between boys and girls is *not significant*, whereas a *significantly* higher rate of boys, as compared to the girls, was selected by the A. C. H. index.

The suitability of the index for assessment of malnutrition has been discussed in detail by Aykroyd *et al.* (1938). In the present study the index has apparently selected a very high percentage of children as compared to the percentage of children diagnosed as malnourished (poor nutrition) either by the naked-eye survey of the physique as a whole or judged by the incidence of clinical signs. It was found that a considerable percentage of children showing clinical signs was not selected by the A. C. H. method. These findings simply support the suggestions made by others that certain amount of modification has to be made in the criterion table compiled by its American authors before the A. C. H. index can be used safely in the study of 'nutrition' of Indian children.

SUMMARY OF FINDINGS.

1. An investigation into the food intake of 177 families consisting of 845 persons was carried out at Jamshedpur in the autumn of 1938.

2. Shortage of calories or quantitative deficiency was noticed in about 28 per cent of the families in the lowest income group.

3. The consumption of oils, fats (as also 'ghee'), and milk was found to maintain a positive correlation with the income of the family.

4. The intake of protective foods, though increasing in quantity with a rise in income, was found to be inadequate even in the highest income group.

5. On the average the amount of total protein in the dietary was adequate but the percentage of protein from animal sources rarely exceeded 12 per cent.

6. Quite contrary to the consumption of the other protective foods, intake of green leafy vegetable was found to maintain a negative correlation with income. This apparent incongruity was due to the fact that the poorer groups of people often grew a few edible creepers inside their huts.

7. About 2,000 children were examined as to their state of nutrition by clinical and A. C. H. methods. The latter method selected an abnormally high percentage of children as compared to the percentage of malnutrition diagnosed by the former. The incidence of dental caries was found to be as high as 30 per cent.

8. Suggestions as to the possible improvements in the dietary have been made.

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APPENDIX.

Distribution of families according to intake per man-value daily of the various proximate principles of food.

Nutrients.	Group I.		Group II.		Group III.		Group IV.	
	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.
Calories :—								
Up to 2,000 ..	3	2·7	..	..	..	..	..	..
„ 2,500 ..	20	18·2	1	2·9	..	..	..	..
„ 3,000 ..	31	28·2	8	22·9	7	36·8	2	15·4
„ 3,500 ..	44	40·0	19	54·2	5	26·3	6	46·2
Over 3,500 ..	12	10·9	7	20·0	7	36·8	5	38·4
Totals ..	110	100·0	35	100·0	19	99·9	13	100·0
Calories from cereals (per cent) :—								
Up to 60 ..	..	..	3	8·6	2	10·5	8	61·5
„ 70 ..	8	7·3	1	2·9	9	47·4	3	23·1
„ 80 ..	25	22·7	18	51·4	3	15·8	1	7·7
„ 90 ..	53	48·2	13	37·1	5	26·3	..	..
Over 90 ..	24	21·8	..	..	..	..	1	7·7
Totals ..	110	100·0	35	100·0	19	100·0	13	100·0
Calories from fats and oils (per cent) :—								
Up to 5 ..	84*	76·3	15	42·9	5	26·3	1	7·7
„ 10 ..	19	17·3	12	34·3	7	36·8	..	..
„ 15 ..	7	6·4	5	14·2	3	15·8	4	30·8
„ 20 ..	..	..	3	8·6	2	10·5	7	53·8
Over 20 ..	..	..	..	..	2	10·5	1	7·7
Totals ..	110	100·0	35	100·0	19	99·9	13	100·0

* Two families have not consumed fats and oils.

APPENDIX—*contd.*

Nutrients.	Group I.		Group II.		Group III.		Group IV.	
	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.
Vitamin A :—								
Up to 1,000 I. U.	48	43·6	12	34·3	9	47·4	7	53·8
„ 2,000 „	25	22·7	13	37·1	5	26·3	3	23·1
„ 3,000 „	13	11·8	3	8·6	1	5·3	..	..
„ 4,000 „	7	6·4	1	2·9	2	10·5	2	15·4
Over 4,000 „	17	15·5	6	17·1	2	10·5	1	7·7
Totals ..	110	100·0	35	100·0	19	100·0	13	100·0
Vitamin B₁ :—								
Up to 600 I. U.	6	5·5	..	..	1	5·3	1	7·7
„ 800 „	31	28·2	9	25·7	3	15·8	2	15·4
„ 1,000 „	55	50·0	11	31·4	8	42·1	5	38·4
„ 1,200 „	14	12·7	14	40·0	5	26·3	5	38·4
Over 1,200 „	4	3·6	1	2·9	2	10·5	..	..
Totals ..	110	100·0	35	100·0	19	100·0	13	99·9
Vitamin C :—								
Up to 50 I. U.	62*	56·4	17	48·6	10	52·6	9	69·2
„ 100 „	25	22·7	11	31·3	6	31·6	4	30·8
„ 150 „	6	5·5	3	8·6	1	5·3	..	..
„ 200 „	5	4·5	1	2·9	..	..	..	..
Over 200 „	12	10·9	3	8·6	2	10·5	..	..
Totals ..	110	100·0	35	100·0	19	100·0	13	100·0

* Three families have not taken anything containing vitamin C.

APPENDIX—*contd.*

Nutrients.	Group I.		Group II.		Group III.		Group IV.	
	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.
Calcium :—								
Up to 0·200 g. ..	21	19·1	1	2·9	..	..	..	..
„ 0·400 „ ..	46	41·8	9	25·7	5	26·3	2	15·4
„· 0·600 „ ..	22	20·0	17	48·6	7	36·8	3	23·1
„ 0·800 „ ..	11	10·0	6	17·1	4	21·1	4	30·8
Over 0·800 „ ..	10	9·1	2	5·7	3	15·8	4	30·8
Totals ..	110	100·0	35	100·0	19	100·0	13	100·1
Phosphorus :—								
Up to 1·000 g. ..	9	8·2	..	..	..	..	..	..
„ 1·200 „ ..	22	20·0	1	2·9	3	15·8	2	15·4
„ 1·600 „ ..	59	53·6	19	54·2	5	26·3	2	15·4
„ 2·000 „ ..	17	15·5	14	40·0	7	36·8	7	53·8
Over 2·000 „ ..	3	2·7	1	2·9	4	21·1	2	15·4
Totals ..	110	100·0	35	100·0	19	100·0	13	100·0
Carbohydrate :—								
Up to 400 g. ..	2	1·8	..	..	..	..	..	..
„ 500 „ ..	17	15·5	1	2·9	2	10·5	2	15·4
„ 600 „ ..	25	22·7	14	40·0	6	31·6	8	61·5
„ 700 „ ..	47	42·7	16	45·7	9	47·4	2	15·4
Over 700 „ ..	19	17·3	4	11·4	2	10·5	1	7·7
Totals ..	110	100·0	35	100·0	19	100·0	13	100·0

APPENDIX—*concld.*

Nutrients.	Group I.		Group II.		Group III.		Group IV.	
	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.	Actual.	Percentage.
Protein :—								
Up to 50 g. ..	15	13·6	..	..	..	..	..	..
„ 60 „ ..	22	20·0	3	8·6	2	10·5	..	..
„ 70 „ ..	24	21·8	6	17·1	2	10·5	2	15·4
„ 100 „ ..	48	43·6	24	68·6	13	68·4	10	76·9
Over 100 „ ..	1	0·9	2	5·7	2	10·5	1	7·7
Totals ..	110	99·9	35	100·0	19	99·9	13	100·0
Animal protein :—								
No intake ..	36	32·7	3	8·6	2	10·5	1	7·7
Up to 10 g. ..	66	60·0	27	77·1	8	42·1	5	38·5
„ 20 „ ..	8	7·3	3	8·6	9	47·4	7	53·8
„ 30 „ ..	..	..	2	5·7	..	..	..	..
Totals ..	110	100·0	35	100·0	19	100·0	13	100·0
Fat :—								
Up to 20 g. ..	64	58·2	10	28·6	5	26·3	1	7·7
„ 40 „ ..	35	31·8	13	37·1	3	15·8	1	7·7
„ 50 „ ..	7	6·4	4	11·4	2	10·5	1	7·7
Over 50 „ ..	4	3·6	8	22·9	9	47·4	10	76·9
Totals ..	110	100·0	35	100·0	19	100·0	13	100·0
Animal fat :—								
No intake ..	35	31·8	1	2·9	2	10·5	1	7·7
Up to 10 g. ..	69	62·7	19	54·3	4	21·1	0	..
„ 20 „ ..	4	3·6	9	25·7	5	26·3	1	7·7
„ 50 „ ..	2	1·8	5	14·3	7	36·8	8	61·5
Over 50 „ ..	..	..	1	2·9	1	5·3	3	23·1
Totals ..	110	99·9	35	100·1	19	100·0	13	100·0

THE COURSE OF EXCRETION OF ASCORBIC ACID IN URINE AFTER ITS INTAKE IN LARGE DOSES.

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THIS investigation was undertaken to find out (*a*) what percentage of the total excess excretion in 24 hours, after an excess intake of ascorbic acid, is given out in the first period of 12 hours and in the following period, (*b*) if there is any relationship between the excretions in these two periods and the total excess excretion in the whole day, which generally indicates the state of saturation of the body, and (*c*) fluctuations if any in the total excess excretion of the vitamin from day to day after the daily intake of an excess of ascorbic acid.

METHOD.

The estimation of vitamin C in urine was made by the usual method with the indophenol dye. Standardized tablets of ascorbic acid were given to the experimented persons, as supplements.

RESULTS.

Tables I, II, and III give the results of experimental studies with regard to points (*a*) and (*b*).

Table I relates to a subject R. K. K. who was in a very low state of saturation, as is evident from a slight increase in the percentage excretion after the

intake of 3,000 mg. of ascorbic acid (viz., from 2 per cent to 18·49 per cent). The ratio between the excretion in the two periods of a day is found to be nearly 60 to 40, the excretion in the first period rising with a higher state of saturation of the body.

TABLE I.

Percentage of excess excretion of vitamin C in different periods after the intake of a test dose by different persons.

Number of days.	Subject.	Amount of intake of vitamin C, mg.	AMOUNT EXCRETED EXCLUDING NORMAL AVERAGE EXCRETION, MG.		PERCENTAGE OF EXCRETION OF VITAMIN C ABOVE NORMAL EXCRETION.		Percentage of total excess excretion in 24 hours after an excess intake of vitamin C.
			1st 12 hours.	2nd 12 hours.	1st 12 hours.	2nd 12 hours.	
1		500	14·6	10·0	60·0	40·0	2·0
2		500	14·0	8·8	61·4	38·6	1·6
3		500	10·47	7·5	58·3	41·7	0·65
4	R. K. K.	500	12·5	7·5	62·5	37·5	4·0
5		500	58·66	33·8	63·4	36·6	18·49
6		500					

TABLE II.

Percentage of excess excretion of vitamin C in different periods after the intake of a test dose by different persons.

Number of days.	Subject.	Amount of intake of vitamin C, mg.	AMOUNT EXCRETED EXCLUDING NORMAL AVERAGE EXCRETION, MG.		PERCENTAGE OF EXCRETION OF VITAMIN C ABOVE NORMAL EXCRETION.		Percentage of total excess excretion in 24 hours after an excess intake of vitamin C.
			1st 12 hours.	2nd 12 hours.	1st 12 hours.	2nd 12 hours.	
1		200	2·46	2·2	52·7	47·3	2·3
2		500	Not examined.	Not examined.	..	..	..
3	G. K. R.	500	211·8	77·2	73·2	26·8	57·8
4		500	172·0	97·3	63·8	36·2	53·8

TABLE III.

Percentage of excess excretion of vitamin C in different periods after the intake of a test dose by different persons.

Number of days.	Subject.	Amount of intake of vitamin C, mg.	Amount excreted excluding normal average excretion, mg.		Percentage of excretion of vitamin C above normal excretion.		Percentage of total excess excretion in 24 hours after an excess intake of vitamin C.
			1st 12 hours.	2nd 12 hours.	1st 12 hours.	2nd 12 hours.	
1		250	30·0	23·2	57·0	43·0	21·2
2		250	90·0	74·9	54·5	45·5	66·0
3	R. N. N.	250	85·7	69·15	55·0	45·0	61·94
4		250	138·0	48·5	74·0	26·0	74·6
5		50	44·0	16·0	73·0	27·0	120·0

Table II relates to a person G. K. R. who was in a fairly high state of saturation, although his initial excess excretion after the administration of the supplement of 200 mg. was very low, viz., 2·3 per cent. This is evident from the fact that after the intake of another 1,000 mg. of ascorbic acid, his total excretion shot up from 2·3 per cent to 57·8 per cent. In his case the difference in excretion between the two periods of a day is not considerable when the excretion was low, but the percentage excretion increased in the first period and accordingly diminished in the second period, though not proportionally, when the percentage of total excess excretion increased.

Table III relates to a person who was in a still higher state of saturation, for his initial excess excretion after the intake of 250 mg. vitamin C was 21·2 per cent which abruptly rose to 66 per cent after the further intake of 250 mg. of vitamin C. The difference of excretion between the two periods of a day was nearly as considerable as in the case of the subject R. K. K. of Table I when the percentage of total excess excretion was low. This difference increased considerably but not proportionately towards the end of the experiment when the percentage of total excess excretion increased.

It is thus obvious that the difference in excretion between the two periods of a day increases, though not proportionally, in a higher state of saturation of the body, insomuch that in the first period of 12 hours, after the intake of a dose of ascorbic acid, a considerably higher percentage of the total excess excretion of the ingested vitamin is eliminated from the body.

Table IV throws a flood of light on point (c). So long as the body is in a low state of saturation, the course of excretion of the ingested vitamin is more or less

even (*vide* No. 2 in the table). While the body begins to be saturated, the excretion mounts up, but seldom becomes constant even while the body is saturated (*vide* Nos. 4 and 5). In No. 5 the fluctuation in excretion after the body was saturated, was between 78·4 per cent and 92·9 per cent and in No. 4 it was between 64·1 per cent and 116·6 per cent. In the latter case, the average percentage of the last two days' excretion was 93·2 which is not very different from the percentage of excretion during the previous two days.

After the body is saturated with vitamin with extra high doses, if the intake be suddenly reduced, the percentage of excess excretion is, as a rule, abruptly increased on the day following the reduced intake (in some cases, the percentage is even above 100) and is then lowered (*vide* No. 7 in Table IV). Kellie and Zilva (1939) also got the same result.

TABLE IV.

Variation in the percentage of excess excretion after the daily ingestion of a supplement of vitamin C.

Number of days.	No. 1. S. R. M.			No. 2. P. R. K.		
	Supplement of ascorbic acid, mg.	Total excretion, mg.	Percentage of excretion of the daily ingested supplement of vitamin excluding normal daily excretion.	Supplement of ascorbic acid, mg.	Total excretion, mg.	Percentage of excretion of the daily ingested supplement of vitamin excluding normal daily excretion.
	Nil	19·1	..	*Nil*	5·6	..
1st	Lemon juice = 49.	22·8	7·4	Orange juice = 105.	13·8	7·79
2nd	Orange juice = 72.	36·9	24·5	do.	13·5	7·5
3rd	do.	62·4	59·75	do.	Not examined.	
4th	do.	54·0	48·1	do.	12·2	6·27
5th	do.	80·0	84·0	do.	14·0	7·9
6th	*Nil*	47·0	28 mg. excreted in excess of normal daily excretion.	..	..	..
7th	..	..	..	..	..	..
8th	..	..	..	..	..	..
9th	..	..	..	..	..	..
10th	..	..	..	..	..	..
11th	..	..	..	..	..	..
12th	..	..	..	..	..	..
13th	..	..	..	..	..	..
14th	..	..	..	..	..	..
15th	..	..	..	..	..	..
16th	..	..	..	..	..	..
17th	..	..	..	..	..	..

TABLE IV—*contd.*

Number of days.	No. 3. G. K. R.			No. 4. N. K. G.		
	Supplement of ascorbic acid, mg.	Total excretion, mg.	Percentage of excretion of the daily ingested supplement of vitamin excluding normal daily excretion.	Supplement of ascorbic acid, mg.	Total excretion, mg.	Percentage of excretion of the daily ingested supplement of vitamin excluding normal daily excretion.
	Nil	11·8	Normal	*Nil*	11·8	Normal
1st	500 crystalline vitamin C.	100	17·7	Orange juice = 168.	11·5	No excess excretion.
2nd	do.	236·4	44·9	do. 200	11·5	do.
3rd	170 crystalline vitamin C + pineapple containing 330.	369	71·4	do.	13·0	0·6
4th	500 crystalline vitamin C.	546·0	106·8	do.	19·45	3·8
5th	..	..	..	do.	21·5	4·8
6th	..	..	..	*Nil*	10·4	Nearly normal.
7th	..	..	..	Orange juice = 200.	26·6	7·4
8th	..	..	..	do.	69·0	28·6
9th	..	..	..	do.	90·0	39·1
10th	..	..	..	Vitamin C tablets = 200.	140·0	64·1
11th	..	..	..	do.	148·0	68·1
12th	..	..	..	do.	Not examined.	
13th	..	..	..	do.	190·0	89·1
14th	..	..	..	do.	190·0	89·1
15th	..	..	..	do.	Not examined.	
16th	..	..	..	do.	245·0	116·6
17th	..	..	..	do.	151·4	69·8

TABLE IV—*contd.*

Number of days.	No. 5. P. K. B.			No. 6. K. P. P.		
	Supplement of ascorbic acid, mg.	Total excretion, mg.	Percentage of excretion of the daily ingested supplement of vitamin excluding normal daily excretion.	Supplement of ascorbic acid, mg.	Total excretion, mg.	Percentage of excretion of the daily ingested supplement of vitamin excluding normal daily excretion.
	Nil	10·5	Normal	*Nil*	19·0	Normal.
1st	Orange juice = 200.	14·85	2·1	500 tablets	10·8	Below normal.
2nd	do.	35·69	12·5	do.	Not examined.	
3rd	do.	92·4	41·0	do.	138·8	23·9
4th	do.	167·4	78·45	do.	300·0	56·2
5th	do.	170·4	79·9	do.	220·0	40·0
6th	do.	188·4	88·9	do.	207·2	37·6
7th	do.	172·0	80·75	do.	276·4	51·5
8th	Vitamin C tablets = 200.	168·4	78·9	do.	238·0	44·0
9th	do.	172·4	80·9	..	..	..
10th	do.	Not examined.		..	..	..
11th	do.	196·4	92·9	..	..	..
12th	do.	184·4	86·9	..	..	..
13th	*Nil*	14·8	Nearly normal.	..	..	..
14th	,,	Not examined.		.	..	..
15th	,,	14·8	Nearly normal.	. ..	..	..

TABLE IV—*concld.*

Number of days.	No. 7. A. K. D.			No. 8. B. D.		
	Supplement of ascorbic acid, mg.	Total excretion, mg.	Percentage of excretion of the daily ingested supplement of vitamin excluding normal daily excretion.	Supplement of ascorbic acid, mg.	Total excretion, mg.	Percentage of excretion of the daily ingested supplement of vitamin excluding normal daily excretion.
	Nil	11·0	Normal	*Nil*	2·4	Normal.
1st	500 tablets	147·2	27·2	500 tablets	3·5	Slight increase in excretion.
2nd	do.	283·2	34·4	do.	3·0	do.
3rd	do.	300·0	57·8	do.	Not examined.	
4th	do.	Not examined.		do.	150·0	30·0
5th	do.	378·2	73·4	200	118·0	59·0
6th	200	Not examined.		200	76·35	38·0
7th	200	218·6	103·8	200	125·0	62·0
8th	50	80·4	138·8	200	128·0	64·0
9th	50	38·0	54·0	200	Not examined.	
10th	50	34·0	46·0	200	162·0	81·0
11th	..	..	..	200	Not examined.	
12th	..	..	..	200	160·0	80·0
13th	..	..	..	200	161·8	81·0

Sometimes the course of excretion may be very much affected by a particular food. Thus, the intake of cabbage by R. N. N. [*vide* Table I, Basu and Ray (1940) this issue] always caused a large increase in the percentage of excretion of vitamin C often beyond 100 per cent. Musulin *et al.* (1938) also noticed a similar marked rise in excretion of vitamin C after the intake of oats and some other substances.

An important fact that has clearly emerged out of examination of Nos. 3, 4, 5, 6, and 7 in Table IV, is that the initial low excretion of ascorbic acid is not an index to the state of unsaturation of his body. This has also been referred to in the previous paper of the present authors (Basu and Ray, *loc. cit.*).

DISCUSSION.

These experiments bring out very clearly that the excretion of ascorbic acid in urine, after the intake of a large dose, is dependent, as has been observed

previously by various workers, upon the state of saturation of the person (*cf.* Tables I, II, and III). When the body is saturated, it is expected there would be an equilibrium between the intake and output of vitamin C. The experimental results given in Table IV show that this is rarely attained. The record of B. D. (No. 8 in the table) is unique, for this is the only case in which the equilibrium has been attained on four consecutive days. In the case of P. K. B. (No. 5 in the table) the percentage of excess excretion shows that although his body was saturated from the fourth day, yet there was no equilibrium between the intake and output even up to the 12th day of the experiment which was then stopped. The case of N. K. G. (No. 4 in the table) also leads to the same conclusion. A noteworthy point in this case is that the excretion suddenly mounted up above 100 per cent on the 16th day and came down on the next day, so that the average excretion of those two days is not very different from that on the 13th or on the 14th day. Howley *et al.* (1936) also noticed similar abrupt changes in excretion during saturation of a person with vitamin C.

When the body is not saturated, the excretion of vitamin C after even a large dose is either not increased at all or only very slightly (*vide* No. 2, first three days in No. 4, and first two days in No. 8 of Table IV). This has also been observed by various workers. The course of increase in blood-ascorbic-acid after the intake of a heavy dose under conditions of saturation and unsaturation of the body requires to be investigated. Like the blood-sugar curve, the blood-ascorbic-acid curve may throw a flood of light on the ascorbic-acid content and metabolism in the body.

The percentage of excretion of ascorbic acid after the ingestion of a heavy dose is generally somewhat greater in the period following the intake than in subsequent periods, but it is particularly so when the body is saturated.

After the intake of a heavy dose the rate of absorption of vitamin C from the alimentary canal would be much greater in the first period on account of its very high concentration therein than in subsequent periods when the concentration would be gradually reduced owing to previous absorption. In consequence of this, the ascorbic acid concentration in the blood and accordingly the urinary excretion would be much greater in the first period than in subsequent periods.

If the tissues, however, remain unsaturated, they absorb with avidity a portion of the excess of ascorbic acid from blood, the greater the absorption the more unsaturated the tissues are. Consequently the urinary excretion in this period does not follow the rate of absorption from the alimentary canal, but depends on the rate of absorption by the tissues. In later periods after the intake of the vitamin, although the rate of absorption from the alimentary canal is reduced, the absorption by the tissues also diminishes, as they are then more saturated than before. Accordingly it is noticed that when the tissues are unsaturated, the difference in excretion between the first period of twelve hours and the subsequent period is not great. When the tissues are more or less saturated, they would naturally withdraw a very small amount of the vitamin from blood. In such circumstances, the urinary excretion follows the rate of absorption of the vitamin from the

alimentary canal, the more closely the greater the saturation of the tissues. This is the reason why the percentage of urinary excretion of the vitamin after its intake is greater in the first period than in the next, when the tissues are more or less saturated.

SUMMARY.

The manner of excretion of the vitamin after its intake in heavy doses was studied and it was found that a higher percentage of the total excess output of the vitamin in the day following the intake was given out in the first period of 12 hours, this percentage rising with an increase in the total excess output, i.e., with the attainment of a higher state of saturation of the body. It was also noticed that the excretion of the vitamin rarely becomes constant and never becomes equal to the amount ingested even when the body is apparently completely saturated.

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OBSERVATIONS ON THE AVERAGE URINARY OUTPUT AND THE STATE OF SATURATION OF BENGALI BOYS WITH RESPECT TO VITAMIN C.

BY

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THE rôle of vitamin C in various phases of human nutrition is well known. Its importance as a respiratory carrier is receiving attention. Its use in increasing resistance to rheumatism and respiratory diseases has been discussed (Harris, 1937). It may have a specific function in stimulating the growth of formative cells in general (Harris, *loc. cit.*). The complement titre value and the vitamin-C content of serum are found to run parallel (Editorial; *Jour. Amer. Med. Assoc.,* 1938). In consideration of these properties of vitamin C its intake by a community and the state of saturation with this vitamin of persons belonging to the community require attention, especially in view of the fact that in the sub-optimal condition of nutrition the various prominent symptoms of deficiency of this vitamin do not appear.

The present inquiry was therefore undertaken (a) to assess the state of saturation with respect to vitamin C of middle-class Bengali boys, (b) to find out the optimum requirements of this vitamin under prevailing conditions of dietary habits entirely different from those of the people of Europe and America, and (c) to discover whether the average normal urinary excretion gives any sure indication of the extent of saturation of the body.

In the present paper detailed observations have been made on the state of saturation of selected boys and on the correlation of the state of saturation with the initial urinary excretion. The optimum requirements will be discussed in a future communication.

(917)

METHOD.

The method followed in this inquiry is a modification of that of van Eekelen (1933) and Johnson and Zilva (1934) and is as follows :—

(*i*) The average output of free and total ascorbic acid excreted in the urine in 24 hours was determined. The previous investigators determined only the output of free ascorbic acid in studies of this nature. Ascorbic acid taken with food may be excreted partly as ascorbic acid, or partly as de-hydro ascorbic acid in urine. Further, urine contains also combined ascorbic acid. The excretion of all these different forms of ascorbic acid has, therefore, been ascertained in some cases under normal conditions and after the ingestion of definite amounts of ascorbic acid so as to find the correlation, if any, between them.

(*ii*) The subject was then given daily supplements of ascorbic acid, either in the form of the juice of fruits rich in ascorbic acid or as standardized tablets of the vitamin, prepared by Hoffmann la Roche. The excess excretion of the vitamin was ascertained daily by deducting his normal average excretion from the total excretion of vitamin C. The administration of the supplement was in the case of certain persons continued till the percentage of excess excretion above normal either became more or less constant (*vide* B. D., R. A., A. K. D., and P. K. B. in the Graph) or rose to 60 or higher up of the ingested amount (*vide* R. N. N., K. P. P., G. K. R., S. R. M., and N. K. G. in the Graph). These persons are then said to have arrived at the upper limit of saturation of their bodies with vitamin C. In other cases the administration was continued till the percentage of excess excretion came up to 30 or slightly more than that (*vide* R. K., P. K., A. R., M. H. C., and A. A. in the Graph). These people, according to Youmann (1937), reached the lower limit of saturation of their bodies with the vitamin. The diet of the subject under investigation remained, as far as possible, constant during the course of the experiment.

(*iii*) The total amount of ascorbic acid ingested as a supplement by a subject during the period of experiment minus the sum total of the excess excretion during the same period gives the amount of ascorbic acid required by him to arrive at the upper limit of saturation of his body with vitamin C in the former case or at the lower limit of saturation in the latter case.

The determination of ascorbic acid in urine by Tillmann's dye presents certain difficulties, for urine contains, besides ascorbic acid, other substances which also reduce this specific dye. To overcome these obstacles Ferrand *et al.* (1937) suggested treatment of the urine with Norite charcoal which, according to them, oxidizes specifically ascorbic acid and no other reducing substance present in urine. The present authors compared the oxidation of urine by Norite with that by hexoxidase prepared from drumstick juice according to Srinivasan's method (1937) but could not substantiate Ferrand's claim of specific oxidation of urinary ascorbic acid by Norite.

Ferrand *et al.* (*loc. cit.*) also suggested the determination of urinary ascorbic acid after precipitation of the other reducing substances by Hg acetate. As these

other reducing substances in normal urine are generally very small in amount this devious method has not been adopted.

GRAPH.

The percentage of excess daily excretion in urine following the ingestion of a supplement of vitamin C.
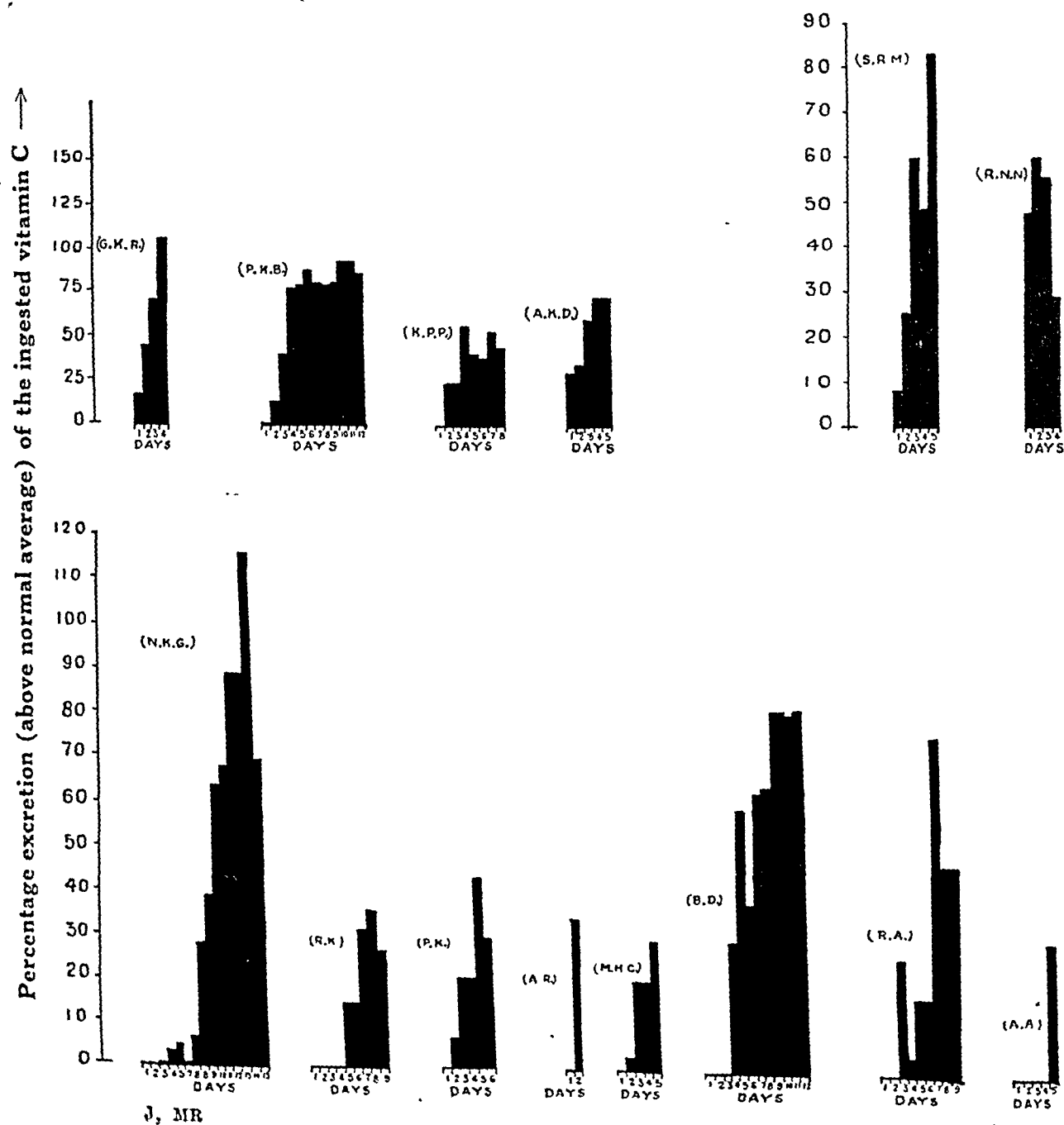
The estimation of de-hydro ascorbic acid was made by the usual technique. The combined ascorbic acid was estimated as follows :—

A definite volume of urine was boiled for 10 minutes in a flask (fitted up with a reflux condenser) in a current of H_2S. Boiling releases ascorbic acid from its combination. The presence of H_2S prevents the aerobic oxidation of both· the pre-existing and released ascorbic acid. It further reduces the de-hydro ascorbic acid to ascorbic acid. The passage of H_2S was continued for five minutes after boiling was stopped. H_2S was then expelled by the passage for about half an hour of a strong current of CO_2. The urine was then titrated with the standardized solution of 2 : 6 di-chlorophenol-indophenol. The titre value gives the sum total of free, de-hydro and combined ascorbic acid.

Another aliquot portion of an equal volume of the same sample of urine was subjected to a current of H_2S for 15 minutes, for the reduction of the de-hydro acid to free ascorbic acid. H_2S was then expelled as before. The urine was then titrated with the dye solution. The titre value gives the sum total of free and de-hydro acid. On deducting the latter value from the former, the amount of combined ascorbic acid was obtained.

In estimating de-hydro ascorbic acid or combined ascorbic acid, H_2S was not passed through urine for more than 15 minutes, as Mack and Tressler (1937) have shown that prolonged treatment with H_2S causes the production of a considerable amount of reducing substances (other than ascorbic acid) in the urine. When these appear in the urine in sufficient amount, they are to be precipitated by Hg acetate or better still by Ba acetate, before the estimation of ascorbic acid by the dye solution is done.

The specimen of urine was collected in the course of the whole day and night for these experiments in bottles containing sufficient glacial acetic acid and M-phosphoric acid so as to make 10 per cent and 3 per cent concentrations, respectively. Toluol was also added to form a continuous layer on the surface. Although the loss of ascorbic acid in urine, collected in H_2SO_4 so as to make a 5 per cent concentration, is found to be much less than when collected in acetic acid and M-phosphoric acid, yet the former method of collection was not adopted in these experiments for the following reasons :—

Before the urine bottles were given out, sufficient amounts of preservatives used to be kept, for the sake of convenience of the subjects, in those bottles so as to make the requisite concentration of the acids when 24 hours' urine was collected therein. If the preservative used be H_2SO_4, the amount of pure sulphuric acid to be kept in the bottle beforehand would be about 75 c.c., so as to make the requisite 5 per cent concentration when 24 hours' urine (which generally amounts to about 1,500 c.c.) is collected. When urine is first passed in such a bottle containing 75 c.c. sulphuric acid, the concentration of sulphuric acid is so high that the organic substances of the urine are partially charred. Consequently, the urine becomes dark in so much that the end-point in the course of the titration with Tillmann's dye cannot be properly detected even with a comparator.

RESULTS OF EXPERIMENTS.

(i) *The normal urinary output of vitamin C in 24 hours.*

The normal daily urinary output of free and total ascorbic acid of 17 boys was estimated. Five of these boys, viz., (1), (2), (3), (4), and (7), in Table III, were taking fruits or vegetables or both during the course of the experiment. Excepting these five boys, all the others had an average daily output of less than 15 mg. of total ascorbic acid, and a majority of them had an output of less than 10 mg. per day with the minimum figure of 2·4 mg. If the excretion of free ascorbic acid be considered, as was done by other workers, then the output is still less, the minimum figure being 1·5 mg. per day. Harris and Ray's (1935) figures vary from 15 mg. to 30 mg. and Villela (1937) found an average of 15 mg. amongst the poor of Rio. In comparison with these figures those for Bengali boys who are living in Calcutta hostels and families and who may, therefore, be considered to be richer than students coming from rural areas, are much lower, showing thereby that they are in a much lower state of saturation than even the poor of Rio.

(ii) *The excretion of de-hydro and combined ascorbic acid.*

The excretion of these different forms of ascorbic acid in relation to free ascorbic acid was studied in detail in the case of three students. The results obtained with these students have been given in Tables I and II.

In Table I, the extract means a soup prepared by the prolonged boiling in water of tomato, beans, cabbages, etc. The subject was taking this soup during the course of the experiment, unless requested not to do so. This soup contained a large amount of ascorbic acid which is not completely destroyed in spite of prolonged boiling. This is evident from the fact that his average daily normal urinary excretion of vitamin C without extract was 28 mg., but with extract 100 mg.

Although cabbage is reported to contain combined ascorbic acid, the soup was free from it, probably because the whole of it was broken up on account of prolonged boiling. The subject, therefore, was not having any combined ascorbic acid.

The features to be noted in this table are :—

(a) The urine was free from de-hydro and combined acids almost throughout the whole period of the experiment.

(b) Although the amount of ascorbic acid voided in the urine was 100 mg. per day, when he was taking the extract, after the administration of large doses of vitamin C in the form of pine-apple juice for several days, the percentage of excess excretion did not exceed 60.

(c) After the administration of cabbage extract as supplement, it was found each time, excepting on the last occasion when he was suffering from fever, that the excretion of the vitamin was much higher than the intake.

It seems therefrom that cabbage causes a washing-out of the vitamin from the body. Musulin *et al.* (1938) have recently shown that oats, unsaponifiable

matter from oat-oil or the volatile fraction from halibut-liver oil causes a marked rise in excretion of vitamin C per day.

The average urinary output per day of free and total ascorbic acid of S. R. M. (Table II) was 10·72 mg. and 19·0 mg., respectively. His body was in a fairly high state of saturation, for he required only 337 mg. of ascorbic acid to attain complete saturation (*vide* Table III). The study of ascorbic-acid metabolism of his body brings out the following important points, as will appear from the table :—

(*a*) After the continued administration of the supplement of vitamin C in the form of orange juice (which is free from combined ascorbic acid) the excretion of free ascorbic acid rose and then remained more or less constant.

(*b*) The excretion of combined ascorbic acid became very high, viz., six times the normal amount and of de-hydro ascorbic acid nearly three times the normal amount..

(*c*) There is no proportionate relationship between the excretion of free ascorbic acid and the other forms of ascorbic acid.

The subject B. K. D. (Table II) normally excreted 41 mg. of free ascorbic acid only (de-hydro and combined ascorbic acid being absent). His body was in a high state of saturation as is proved by the fact that after the administration of pine-apple juice containing 385 mg. of ascorbic acid, the percentage of excretion of his excess intake was nearly 60 on the first day. The excretion of de-hydro and combined acid, curiously enough, jumped up on the second day, then went down and became more or less constant on other days, and showed no relationship to the output either of free or total ascorbic acid. The constancy of excretion is greater in the case of de-hydro than in the case of combined acid.

It would be noticed that of the three subjects whose excretion of de-hydro and combined ascorbic acid was determined, two, viz., R. N. N. and B. K. D. (Tables I and II), passed urine containing normally only free ascorbic acid. The urine of S. R. M. (Table II) contained besides free ascorbic acid other forms of ascorbic acid as well. R. N. N. was given large doses of pine-apple juice and then cabbage extract. His body became saturated with vitamin C, but there was no excretion of de-hydro and combined acid at any stage. B. K. D. was also given pine-apple juice, but in his case both these forms of ascorbic acid appeared in the urine on the ingestion of this juice, and did not disappear after its discontinuance, even when the excretion of total ascorbic acid became very low. Further, there was no relationship between the excretion of these forms of ascorbic acid and the total ascorbic acid or free acid. In the case of S. R. M., after the administration of orange juice which contains only free ascorbic acid, the excretion of combined ascorbic acid rose more or less generally with the excretion of total ascorbic acid, but the excretion of de-hydro acid did not bear any relationship to the excretion of total acid. Thus, these studies did not throw any light on the excretions of these two forms of ascorbic acid, excepting that possibly there is no relationship between their excretion and the excretion of free ascorbic acid.

From Table II, it would be found that the ratio of de-hydro to free ascorbic acid lies between 0·05 and 0·4, but according to Bruno and Giuseppa (1937) it lies

between 0·08 and 0·7. The ratio of combined ascorbic acid to the free acid lies between 0·05 and 1·0, but if the values of combined ascorbic acid excreted by S. R. M. on the fourth and sixth days be rejected as being abnormally high, then the range of the ratio is between 0·05 and 0·66 which closely resembles that of the de-hydro to free ascorbic acid.

The abrupt fall of excretion of these different forms of ascorbic acid in the case of B. K. D. as shown in Table II on the ninth day was due to the fact that the subject who had been taking a constant amount of certain fruits all throughout the experiment, even when the supplement was given to him, suddenly stopped taking them.

(iii) The state of saturation of Bengali boys.

The results of experiments are given in Table III. Four Mohammedan boys, two laboratory bearers, and eleven Hindu boys, were examined. None of them showed any signs of scurvy, although some of them were in a very low state of saturation with this vitamin. The maximum deficit amongst the persons examined was 3,247 mg., i.e., much lower than 5,000 mg., when definite symptoms of scurvy are said to appear (Gander *et al.*, 1936). Analysis of the results summarized in the table reveals the following interesting points :—

(*i*) Persons excreting daily in urine more than 40 mg. of ascorbic acid are either completely or nearly completely saturated with the vitamin (*vide* subjects 1 and 2).

(*ii*) Persons excreting daily less than 30 mg. of ascorbic acid are in various degrees of saturation and no proportional relationship exists between the excretion of ascorbic acid in the urine and the state of saturation of the persons. Thus, the subject A. R. (No. 13), having an excretion of only 4 mg. of total ascorbic acid (or 2·5 mg. of free ascorbic acid), required 1,664 mg. to arrive at the lower state of saturation of the body, whereas the subject M. H. C. (No. 14), with an excretion of 3 mg. of total acid or 2·3 mg. of free acid, required 3,219·6 mg., i.e., nearly double the former amount, for arriving at the same state of saturation. Again, R. K. (No. 10), having an excretion of 14·7 of total ascorbic acid, i.e., nearly five times the excretion of No. 14, needed 3,247 mg., i.e., a little more than the amount of ascorbic acid required by the latter for arriving at nearly the same state of saturation.

(*iii*) Persons excreting less than 10 mg. of total ascorbic acid required, as a rule, more than 2,000 mg. to arrive at a lower state of saturation of the body.

(*iv*) Of the 17 persons examined, two were saturated, two were nearly saturated, for they required less than 500 mg. to arrive at the higher state of saturation, one needed a little over 500 mg. for full saturation and all the others required over 1,000 mg. for arriving at the saturated condition. Amongst the last group of 12 persons nearly

seven (including the subject No. 9 whose body may be taken to be very deficient in vitamin C, as he showed a very slight increase in urinary excretion after the ingestion of 525 mg. of vitamin C) required over 2,000 mg. for arriving at the lower state of saturation of the body with the vitamin. It may, therefore, be mentioned that over 70 per cent of the subjects examined (i.e., 12 persons out of 17 examined) are in an unsaturated condition with regard to this vitamin.

(v) The urinary output of ascorbic acid became generally normal about 24 hours after the supplement was stopped.

In ascertaining the amount required to arrive at the higher state of saturation of the body it was found that the percentage of excretion of the excess intake of the vitamin suddenly jumped up from a lower figure to nearly 60 per cent or much above it and that although it fluctuated within a wide range in some cases, the average excess excretion during saturation was more or less constant in all such cases in which the excess intake was maintained for a number of days (*cf.* Graph). In two of these cases, viz., those of B. D. and P. K. B. (Nos. 15 and 6 in Table III), the excretion may be said to have been remarkably constant. In some of these cases the excess excretion suddenly mounted up on a day and went down to a sufficiently lower figure on the following day so that the average excretion remained nearly constant (*vide* N. K. G. and R. K. in the Graph).

TABLE I.

Variations in the ascorbic acid excretion in urine after its ingestion in different doses (subject—R. N. N.).

Number of days.	Supplement.	Excretion of vitamin C in mg.				Percentage of excretion of the daily ingested ascorbic acid excluding normal daily excretion.	Amount of total excretion of ascorbic acid excluding normal daily excretion.
		Free.	De-hydro.	Combined.	Total.		
1	Nil (without extract).	28	Nil	Nil	28·0	..	..
2	Nil (with extract).	90	10·0	Nil	100·0	..	..
3	Pine-apple = 266 mg. of ascorbic acid.	226	Nil	Nil	226·0	47·2	126·0
4	do.	258	Nil	Nil	258·0	59·2	158·0
5	do.	249	Nil	Nil	249·0	55·8	149·0

TABLE I—*concld.*

Number of days.	Supplement.	EXCRETION OF VITAMIN C IN MG.				Percentage of excretion of the daily ingested ascorbic acid excluding normal daily excretion.	Amount of total excretion of ascorbic acid excluding normal daily excretion.
		Free.	De-hydro.	Combined.	Total.		
6	Pine-apple = 266 mg. of ascorbic acid.	175	*Nil*	*Nil*	175·0	28·1	75·0
7	*Nil*	95 (Normal)	*Nil*	*Nil*	95·0	..	..
8	Cabbage containing free = 36·9 mg. and total 59·0 mg. vitamin C.	138	*Nil*	*Nil*	138·0	64·4	38·0
9	Cabbage containing total ascorbic acid = 34 mg.	206	*Nil*	*Nil*	206·0	Abnormally large.	106·0
10	*Nil*	84 (Below normal)	*Nil*	*Nil*	84·0	..	..
11	Cabbage (free = 36·0 mg. and total = 50·6 mg. ascorbic acid).	138	*Nil*	*Nil*	138·0	76	38·0
12	*Nil*	276 (Abnormally high)	*Nil*	*Nil*	276·0	..	176·0
13	*Nil* (stopped taking the extract).	191 (Abnormally high)	*Nil*	*Nil*	191·0	..	163·0
14	*Nil*	118 (Abnormally high)	*Nil*	*Nil*	118·0	..	90·0
15	*Nil*	30 (Approximately normal)	*Nil*	*Nil*	30·0	..	..
16	Cabbage = 96·3 mg. of ascorbic acid.	136	*Nil*	*Nil*	136·0	More than 100	108·0
17	*Nil*	32 (Almost normal)	*Nil*	*Nil*	32·0	..	4·0
18	Cabbage = 23·5 mg. total ascorbic acid.	16	*Nil*	*Nil*	16·0	Below normal	..

TABLE II.

Excretion of free, de-hydro, and combined ascorbic acid.

Number of days.	Name of subject.	Supplement taken daily.	Excretion of free ascorbic acid in mg.	Excretion of de-hydro ascorbic acid in mg.	Excretion of combined ascorbic acid in mg.	Excretion of total ascorbic acid in mg.	Ratio of de-hydro : free ascorbic acid.	Ratio of combined : free ascorbic acid.
1	S. R. M.	Nil	10·72	2·5	5·9	19·1	0·23	0·055
2	do.	Orange juice = 49 mg. ascorbic acid.	17·3	Nil	5·5	22·8	..	0·31
3	do.	Orange juice = 72 mg. ascorbic acid.	25·5	7·4	4·0	36·9	0·29	0·15
4	do.	do.	30·0	2·4	30·0	62·4	0·08	1·0
5	do.	do.	30·0	4·0	20·0	54·0	0·13	0·66
6	do.	do.	33·3	6·0	40·7	80·0	0·18	1·2
7	do.	Nil	30·0	7·0	10·0	47·0	0·23	0·33
1	B. K. D.	,,	41·0	Nil	Nil	41·0	..	..
2	do.	Pine-apple juice = 385 mg. ascorbic acid.	110·0	20·0	35·0	165·0	0·18	0·31
3	do.	do.	380·0	42·0	53·0	475·0	0·11	0·13
4	do.	do.	400·0	20·0	20·0	440·0	0·05	0·05
5	do.	Nil	175·0	25·0	15·0	215·0	0·14	0·08
6	do.	,,	150·0	18·0	19·5	187·0	0·12	0·13
7	do.	,,	60·0	20·0	20·0	100·0	0·33	0·33
8	do.	,,	108·0	22·0	14·0	144·0	0·19	0·12
9	do.	,,	7·5	3·0	2·6	13·1	0·4	0·34

TABLE III.

Ascorbic acid excretion in urine and the state of saturation.

Number of days.	Name of subject.	Average normal daily vitamin-C excretion in mg. (free).	Average normal daily vitamin-C excretion in mg. (total).	Total amount of vitamin C in mg. ingested before the saturation point was reached.	Total amount of vitamin C in mg. excreted over the normal average daily excretion up to the saturation point.	Amount of vitamin C in mg. required to bring to the saturation point.	Amount of vitamin C in mg. required to bring to the lower limit of saturation.	Percentage of excretion of the ingested amount of vitamin C at the point which was taken as the saturation point.	Time required to bring to the initial normal rate of vitamin-C excretion after stopping the supplement.	Characteristics in the dietary habit (if any) and the nature of the diet.
1	B. K. D.	41·0	41·0	385	285	100	..	60·0	More than 24 hours.	Fruit consumer. Hostel diet. Hostel diet. His daily tiffin consists of 3 guavas.
2	N. C. R.	55·3	55·3	The subject was in a fully saturated condition. Excreted 100 per cent of the ingested amount of ascorbic acid on the first day.				..	..	Fruit consumer. Did not take fruits during the experiment. Mess diet.
3	S. R. M.	10·72	19·1	337	160·9	176·4	102·0	84·0	More than 24 hours.	Average Bengali family diet. Daily took an extract of boiled cabbage, tomato, and beans.
4	R. N. N.	28·0 100 (with extract).	28·0 (without extract) 100 (with extract).	532	284·0	248·0	..	59·2	Within 24 hours.	Average Bengali diet. Daily took an extract of boiled cabbage, tomato, and beans.
5	G. K. R.	10·4	11·6	1,821·0	1,205	616·0	..	106·8	..	Average Bengali diet. Not in the habit of taking fruits regularly. Takes greens.
6	P. K. B.	8·8	10·5	1,400·0	400·0	1,000·0	550·0 mg. vitamin C to bring to 41 per cent excretion.	88·9	After two days.	Takes a little fruit daily but the fruits were poor in vitamin-C content. Average Bengali diet.
7	K. P. P.	19·0	19·0	2,000·0	401·0	1,599·0	..	56·2	..	Average Bengali diet. Takes plenty of greens every day.

TABLE III—concld.

Number of days.	Name of subject.	Average normal daily vitamin-C excretion in mg. (free).	Average normal daily vitamin-C excretion in mg. (total).	Total amount of vitamin C in mg. ingested before the saturation point was reached.	Total amount of vitamin C in mg. excreted over the normal average daily excretion up to the saturation point.	Amount of vitamin C in mg. required to bring to the saturation point.	Amount of vitamin C in mg. required to bring to the lower limit of saturation.	Percentage of excretion of the ingested amount of vitamin C at the point which was taken as the saturation point.	Time required to bring to the initial normal rate of vitamin-C excretion after stopping the supplement.	Characteristics in the dietary habit (if any) and the nature of the diet.
8	A. K. D.	7·0	11·0	2,500·0	964·0	1,536·0	364·0	73·4	Normal within 24 hours.	Average Bengali diet. Formerly used to take fruits.
9	P. R. K.	5·6	5·6	..	..	After the ingestion of 525·0 mg. vitamin C only 8·0 per cent excreted. The experiment had to be discontinued as the subject left for home suddenly.			..	Poor diet of a bearer.
10	R. K.	12·4	14·7	3,500·0	253·0	3,247·0	2,957·0	36·0	Within 24 hours.	† Poor class of diet.
11	P. K.	8·76	9·66	2,600·0	341·0	2,259·0	2,259·0	30·0	..	‡ do.
12	N. K. G.	5·9	11·8	3,000·0	1,022·0	1,978·0	497·0 to bring to 28·6 per cent excretion.	93·2	..	Not in the habit of taking fruits. Hostel diet.
13	*A. R.	2·5	4·0	2,000·0	336·0	1,664·0	1,664·0	34·0	Within 24 hours.	Hostel diet. No fruit intake.
14	*M. H. C.	2·3	3·0	3,500·0	280·4	3,219·6	3,219·6	30·0	..	‡ do.
15	B. D.	1·5	2·4	3,200·0	749·0	2,410·0	1,850·0	81 for four consecutive days.	Within 24 hours.	Average Bengali diet. No fruits.
16	*R. A.	3·8	5·6	2,700·0	318·0	2,382·0	1,375·0	46·0	..	Hostel diet. No fruit.
17	*A. A.	5·8	5·8	2,500·0	145·0	2,359·0	2,359·0	30·0	..	do.

* Mohammedan students.   † Aged about 40 years.   ‡ An old man aged 55 years.

DISCUSSION.

(i) *The normal daily urinary output of vitamin C of middle-class Bengali boys.*

It has been shown previously that the average daily output of middle-class Bengali boys is much lower than either Harris's (*loc. cit.*) or Villela's (*loc. cit.*) figures. In the case of some of these boys the output was very low, e.g., in one case the output of free ascorbic acid was only 1·5 mg. per day, a value which was obtained by Cohen and Nisenson (1937) and others in cases of scurvy.

The subject B. D. whose excretion was lowest, although apparently healthy, had bleeding gums and dental caries and was suffering from pyorrhœa. Nevertheless, not only this subject but also two others, viz., M. H. C. and A. R., whose excretion was as low as was noticed by others in patients suffering from scurvy, had no definite symptoms of that disease. Naturally, therefore, it is open to question whether even a very low urinary output is a definite indication of deficiency of vitamin C in the body, especially when it is noticed that these three very low vitamin-C-excreting subjects required widely different amounts of the vitamin for attaining the lower state of saturation of their body, such as 1,664 mg. by A. R., 1,850 mg. by B. D., and 3,220 mg. by M. H. C. Their deficit is less than 5,000 mg. which, according to Gander *et al.* (*loc. cit.*), corresponds with declared scurvy. Kellie and Zilva (1939) have recently shown that by reducing the daily dose of ascorbic acid from 50 mg. to 30 mg. the urinary excretion of a person falls down almost to zero, although the 'saturation test' shows that he is not far removed from it. In discussing the relationship of the low urinary output of vitamin C to scurvy, two questions deserve special consideration. The first is that if vitamin C is excreted through the sweat, as has been proved by some workers (Wright, 1936 ; Bernstein, 1937), the output through the urine is lowered and the greater the formation of sweat, the less would be the output through urine. In such a case, the urinary output alone of the vitamin cannot possibly be an index to its content in the body. The experiments with the above subjects of very low urinary output were performed during summer in Calcutta, of which the moist heat causes an excessive amount of sensible perspiration and this might be the reason why their urinary output was low. The second point is that there may be destruction of the vitamin in urine before it is voided, and this destruction may be considerable when the urine is neutral and more so when it is alkaline (Hawley, 1936).

(ii) *Excretion of de-hydro and combined ascorbic acid.*

In discussing this point, evidences have been adduced to show that the excretion of these substances is not apparently connected with the output of free ascorbic acid and, therefore, with the ascorbic acid metabolism.

SUMMARY.

1. The average daily urinary output of free ascorbic acid and total ascorbic acid of 17 persons, mostly students living in hostels or families, has been determined. The output was very low in several cases, the lowest figure for free ascorbic

acid being 1·5 mg. per day. Excepting five boys who were taking fruits during the course of the experiment, all the others had an excretion of total ascorbic acid of less than 15 mg. per day, and of free ascorbic acid of less than 13 mg. per day. In spite of such a low daily output of vitamin C symptoms of scurvy could not be detected in any case.

2. The excretion of de-hydro and combined ascorbic acid of some of these boys was ascertained and compared with that of free ascorbic acid both under normal conditions and during a period of excess intake of vitamin C. It was found that the excretion of the former has no apparent relationship to that of the latter, but that when all three are excreted in the urine, the ratio of de-hydro to free ascorbic acid and of combined to free acid lies within a certain range which is almost similar in both cases.

3. The state of saturation of these 17 persons was also assessed. It was found that, excepting five boys who were taking fruits during the course of the experiment, all the others required over 1,000 mg. to arrive at a saturated condition of the body and six required over 2,000 mg. before becoming saturated.

In comparing the state of saturation of these persons with their average daily output of vitamin C, it was noticed that there is no proportional relationship between the average excretion of the vitamin and the state of saturation, unless the excretion is above 30 mg. or 40 mg. per day.

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ON THE COMPOSITION OF SWEAT OF THE INDIANS.

BY

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The part played by the sweat glands in regulating the composition of blood plasma under normal conditions is comparatively insignificant in contrast with that of the kidneys and the lungs ; but there are conditions when the excretory functions of the sweat glands might have important physiological bearings. Moss (1923) pointed out that with continued profuse sweating a great loss of chlorides takes place resulting in cramps of muscles in the body. When the normal function of the kidney is more or less impaired and there is retention of nitrogen the sweat glands may to a limited extent supplement their action in eliminating the excess of nitrogen. Pemberton *et al.* (1929) showed that in the majority of cases of nephritis the nitrogen in the sweat is abnormally high, indicating that sweating aids in nitrogen excretion. Again, while normally small amounts of urea are found in the sweat, it is stated that in uræmia the sweat glands may assume some of the functions of the kidneys and excrete urea in such amounts that the skin is covered with crystals. The sweat glands also constitute an important mechanism in the regulation of body reaction and protect the kidneys from being exposed to the action of large quantities of acids for long periods. This is demonstrable in the excretion of a large quantity of lactic acid in the sweat of persons doing vigorous muscular work.

It has been stated that the composition of sweat of races living in the warm and lurid climate of the tropics differs from that obtained from those living in

temperate climes. Some have gone so far as to say that the natives of the tropics have a greater concentration of the sweat glands per unit area than the European settlers.

It is not unlikely that difference in physical and environmental conditions would have some influence on the activity of the sweat glands and will be reflected to a certain extent on the composition of the sweat. It is also doubtful whether the perspiration induced in a native of a temperate climate by putting him in an artificial environment—a hot chamber for instance—will have the same composition as that which is produced freely in a native of the tropics. It appeared to us to be of interest to determine the composition of sweat amongst Indians with respect to some of the principal constituents and compare it with those found by other workers in tropical and sub-tropical climates.

ANALYSIS OF SWEAT SAMPLES.

Reaction.—Conflicting data are reported as to the reaction of sweat. Some report it to be alkaline when shed, while others have found it to be acid. Heuss (quoted by Talbert, 1919) states that while sweat is normally acid on profuse sweating, however, it rapidly passes to neutral and finally to alkaline reaction. This is apparently confirmed by McConnell and Houghton (1923) who found that perspiration becomes more alkaline as sweating continues. Marchionini (1929) states that the combined perspiration collected from a person in a hot-bath chamber is invariably acid (pH 4·6 to pH 6·5). In severe industrial work in a hot environment the reaction on the other hand is reported to be alkaline (pH 7·8 to pH 8·0).

Whitehouse (1935) stated that the composition of sweat was considerably influenced by substances given off from the general surface of the epidermis. Thus, the reaction of samples collected after very careful washing of the skin was alkaline (pH 7·2 to pH 7·8) ; without this thorough washing the sweat was acid (pH 4·4) and this acidity was communicated to the sweat by the skin surfaces. It appears therefore that the reaction of the sweat depends to a great extent upon the conditions under which the sweat is collected.

In our experiments the sweat was collected as it was shed, in the majority of cases, except in a few where the surface of the skin was rubbed with moist linen prior to the collection of sweat. A reference to the Table will show that the reaction is mostly on the acid side (pH 4·6 to pH 5·9) and a preliminary cleaning of the skin surface prior to the collection of sweat does not make any difference in the reaction.

Chloride content.—Widely varying results are also reported with regard to the chloride content. Moss (*loc. cit.*) reported a variation from 0·118 to 0·325 per cent, Talbert and Haugen (1927), 0·43 to 0·83 per cent, and Barney (1926) from 0·188 to 0·649 per cent with an average of 0·31 per cent. McSwiney (1934) as a result of very careful experiments has fixed the average value to be 0·37 per cent in males and 0·3 per cent in females. These widely-varying results are stated to be due to

methods of collection and to a certain extent the chloride excretion may also depend upon the temperature. Cuthbertson and Guthrie (1934) observed that increase in salt intake had no constant effect on the output of chloride in the sweat but an increase of temperature, the chloride intake remaining constant, caused an increased output of chlorides. Whitehouse (1931, 1935) found that the chloride concentration of the sweat of different individuals varied markedly. In one subject it was increased by severe muscular work, but not to the same extent as when the same increase in body temperature was produced with the subject at rest. The chloride concentration increases but little during the performance of a moderate amount of exercise but increases markedly with time when the subject is at rest and perspiration is due simply to the humidity.

On referring to the table it would appear that the chloride content calculated as NaCl in our series varies from 0·073 to 0·844 per cent (average 0·44 per cent). There appears to be no significant relation between the chloride content and moderate muscular work. The sweat at rest may have a high chloride content, while after muscular work it may have low values and vice versa.

Ammonia, urea-N, etc.—Talbert, Finkle and Katsuki (1927) reported that ammonia-N varied from 5 mg. to 35 mg. per 100 c.c. and there was a correlation of 41 per cent between ammonia nitrogen and urea nitrogen of the sweat. McSwiney (*loc. cit.*) found the ammonia-N to vary from 2·55 mg. to 7·0 mg. (average 4·7 mg.) per 100 c.c. and the urea-N from 11·10 mg. to 32·92 mg. (average 21·44 mg.) per 100 c.c. In our experiments the NH_3-N is found to vary between 7·5 mg. to 34·2 mg. (average 19·3 mg.) per 100 c.c. and urea-N from 10·4 mg. to 61·5 mg. per cent with average of 30·6 mg., so that both the ammonia and the urea-N figures are somewhat higher than what is reported by other workers. We, however, were not able to find any correlation between the NH_3-N and the urea-N values.

The divergence of opinion recorded in the literature as to the H-ion-concentration, NH_3-N, etc., can, to some extent at least, be accounted for by the readiness with which perspiration is decomposed even at the ordinary temperature by the bacteria normally present in the perspiration.

SUMMARY AND CONCLUSIONS.

1. Analyses of samples of sweat collected from several healthy Indians living in and around Calcutta show somewhat different values from those obtained by other workers in tropical and temperate climates.

2. The reaction of the sweat was invariably found to be acid, the pH varying from 4·6 to 5·9 (average 5·05).

3. The chloride content varied from 0·073 to 0·805 per cent with an average at 0·387 per cent.

4. The ammonia-N varied from 10·9 mg. to 34·2 mg. and the urea-N from 10·4 mg. to 61·5 mg., per cent; the average values being 18·17 mg. and 29·2 mg., respectively.

TABLE.

Sweat analysis.

Number of cases.	Ammonia-N mg. per 100 c.c.	Uren-N mg. per 100 c.c.	Percentage of NaCl.	pH value.	REMARKS.
1	..	..	0·479	..	Sweat at rest.
2	..	..	0·013	..	
3	..	..	0·206	..	
4	..	..	0·206	..	
5	..	..	0·370	..	Rest.
6	..	..	0·370	..	After exercise.
7	..	..	0·370	..	,, ,,
8	25·0	..	0·370	..	
9	21·7	..	0·261	..	
10	34·2	31·4	0·206	..	Freshly collected in a closed room.
11	23·8	24·2	0·206	..	After exercise.
12	17·2	27·6	0·088	..	,, ,,
13	29·4	36·3	0·522	..	At rest.
14	29·4	42·1	0·522	..	,,
15	30·3	29·2	0·522	..	,,
16	11·3	32·0	0·196	..	After a walk.
17	15·1	61·5	0·414	..	At rest.
18	13·1	50·0	0·206	..	,,
19	16·12	31·5	0·805	4·6	,,
20	..	..	0·690	4·8	After exercise.
21	17·2	21·9	0·587	4·6	In hot chamber.
22	11·9	27·9	0·653	5·1	After exercise.
23	12·5	29·6	0·170	5·1	After a walk.
24	14·3	22·9	0·587	4·7	After light exercise.
25	7·5	10·7	0·750	5·7	After a walk.
26	10·9	10·4	0·522	4·6	,, ,,
27	17·2	27·9	0·630	4·9	,, ,,

Sweat analysis—concld.

Number of cases.	Ammonia-N mg. per 100 c.c.	Urea-N mg. per 100 c.c.	Percentage of NaCl.	pH value.	REMARKS.
28	18·5	28·5	0·414	5·3	At rest.
29	14·2	25·0	0·098	5·1	In a closed room. The skin rubbed with moist linen before the collection of sweat.
30	15·6	24·4	0·286	5·9	do.
31	13·2	25·2	0·414	5·1	do.
32	16·8	23·2	0·206	4·9	..
33	..	..	..	5·1	..
34	..	..	..	5·2	..
35	..	..	..	5·2	..

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SOME OBSERVATIONS ON THE LIPOID CONTENT OF THE BLOOD IN EPIDEMIC DROPSY.

BY

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IN view of the extensive studies which have been made in recent years on the chemical and cytological changes in the blood in epidemic dropsy it would appear to be opportune to review the present position of our knowledge and to complete the information by the presentation of the results of further observations.

Roy (1927) made a more or less comprehensive investigation into the general and biochemical blood picture of persons suffering from epidemic dropsy. He found a marked diminution of the number of erythrocytes from that of the normal in almost all the cases. The percentage of hæmoglobin was also lower and the colour index showed marked anæmia of the secondary type. The plasma volume was found to be higher and the cell volume consequently lower than the normal, pointing to a distinct hydræmic condition of the blood. The non-protein nitrogen, urea and creatinine content of the blood did not show any pathological deviation. The only abnormality suggesting retention of nitrogenous metabolites is the high figure obtained in the uric-acid values. Roy doubts whether this apparent increase really represents an actual increase in the uric-acid content.

The usual diet of Bengalis, as compared with Europeans, is relatively poor in purin bodies. One would, therefore, expect rather lower values in the uric-acid content, but much higher values are obtained even in severe cases. The absence of renal involvement on a relatively poor purin diet and absence of any data suggesting an extensive break-down of the nuclear materials of the body cells, Roy observes,

make it very difficult to decide if the figures obtained really represent uric acid. A marked reaction for indican in the urine is a common feature of the disease. Roy suggests that a part of the phenols formed by the putrefactive changes of proteins in the intestines is absorbed into the blood and the presence of these bodies may explain the appraently higher blood uric-acid content as the phenols produce a similar colour with the uric-acid reagent as does uric acid itself. It may be that increase of the uric acid is real because when there is excess of carbohydrate and little fat in the diet, as is usual with the Bengali diet, the formation of purin bodies from carbohydrate may be stimulated (Wright, 1934). Again, a transient œdematous condition of the skin, subcutaneous tissue and mucous membrane is seen in œdemas of the angioneurotic type. Results obtained after atophan administration led Hyslop (1926) to think that it was connected with uric-acid retention. In this connection it is of interest to recall the observations of Lukis (1908) who considered epidemic dropsy to be of the nature of angioneurotic œdema closely allied to urticaria and originating from some sepsis.

The chloride content of the blood was, however, found by Roy (*loc. cit.*) to be higher than normal in the majority of cases. The retention of chlorides in these cases with œdema is significant. It recalls to our mind the blood picture as obtained in hydræmic nephritis, where there is retention of chloride without appreciable retention of nitrogenous waste products ; but the absence of any renal involvement and of copious albuminuria puts epidemic dropsy into a separate class. The calcium figures were rather irregular varying from 6·09 mg. to 15 mg. per 100 c.c., but Roy points out that most of the cases had calcium in some form or other before he could examine the blood ; the results in the untreated cases were decidedly lower than normal. Denis and Corley (1925) and Halverson, Mohler and Bergein (1917) maintained that no increase of Ca content of blood serum in animals was possible by supplementing food with calcium or calcium rich food and this is in accord with our observations (unpublished) with respect to human beings. The continued administration of Ca salts in various forms with or without parathyroid causes no appreciable increase of serum calcium content of human beings. Peters and Eiserson (1929) claim that pathological variations in serum calcium cannot be evaluated unless simultaneous determinations of inorganic phosphorus are made. It is therefore difficult to assess the real significance of these observations. Roy *et al.* (1933) confirmed the findings of Roy (*loc. cit.*) in all essential points. The cholesterol content varied from 89 mg. to 166 mg. (average 131·0 mg. per 100 c.c.). Roy considered these figures to be markedly lower than normal values. These figures however fall within the normal range for Indians as obtained by Boyd and Roy (1928) and in fact the average is somewhat higher than that obtained by these workers (116 mg. per 100 c.c.).

The study of the protein fractions as well as some of the physical properties of blood sera of patients suffering from epidemic dropsy, however, furnishes some very interesting data. The total nitrogen and consequently the protein value was found by Roy to be much below the normal though the serum protein was near about the normal in the majority of cases. The fact that in spite of anæmia and hydræmia which are almost constant features of this disease, the serum proteins

retained their normal value, indicated that it was the diminished number of erythrocytes that was mainly responsible for the diminished total N-value. The albumin : globulin ratio was found to have altered to a marked degree. The albumin content was found to be much decreased and the globulin fraction was much higher than the normal.

Chopra *et al.* (1935) found that in the sera of patients suffering from epidemic dropsy both relative viscosity and surface tension were below their normal values. The pH remained unaltered, while the buffer action was diminished to a certain extent. As regards the protein fractions, their findings were in agreement with those of Roy (*loc. cit.*), the only difference being that this worker found still more marked changes in the albumin fraction. These authors have also sought to explain some of the symptoms of epidemic dropsy in the light of changes of protein fractions as well as of other physical properties of blood sera already alluded to. The three main factors which govern the fluid exchange between the blood and the tissues are : (1) the hydrostatic pressure of the blood and this in turn depends upon its viscosity. The lowered viscosity as observed in these cases increases the hydrostatic pressure which tends to drive fluid into the tissues, (2) the osmotic pressure of the serum colloids tends to draw fluid into the blood, the albumin fraction being responsible for this. A fall in this protein as is observed in this condition therefore lowers the osmotic pressure and cannot attract fluid into the blood as efficiently as in normal subjects, and (3) the increase of capillary permeability as is observed in epidemic dropsy also influences greatly the fluid exchange in the body.

From this it would appear that in epidemic dropsy almost all the factors that control the fluid exchange between the blood and the tissue become disturbed and favour the expulsion of fluid from the blood into the tissues.

That the cholesterol and lecithin in the blood undergo significant changes in some of the dropsical conditions and in anæmia is well known. A close relation is said to exist between lecithin and cholesterol in the system ; if one increases the rise is followed closely by the other lipoid. It is believed that this ratio has much to do with the water balance of the body. This theory was put forward by Schæffer (1914) and elaborated on with *in vitro* experiments by Degkwitz (1933). It was considered possible that simultaneous determination of the lecithin and cholesterol content of the blood might throw some light into the disturbed water-balance found in this condition and investigations on the subject were accordingly carried out on cases selected from those attending the Carmichael Hospital for Tropical Diseases, Calcutta.

Cholesterol was estimated according to a slight modification of Bloor's technique (1922) and lipoid-P according to the procedure laid down by Chopra and Roy (1936). In some of the cases the distribution of both lecithin and cholesterol between the cell and plasma was also worked out. The results are given in the following Table. For the sake of convenience the cases have been grouped under the following heads :—

(*a*) Acute cases.

(*b*) Chronic and recurrent cases.

(c) Cases with marked erythema legs.
(d) Cases with sarcoids.
(e) A few cases which do not come under designation epidemic dropsy were taken from beggar class persons who had subsisted on insufficient diet for a long time and who did not exhibit other characteristics of epidemic dropsy and may more aptly be classified as nutritional œdema.
(f) Two cases of sub-acute glomerular nephritis which were encountered in the course of this investigation have also been worked out.

TABLE.

Cholesterol and lecithin content of blood.

(a) *Acute cases.*

Case number.	Age.	CHOLESTEROL.			LECITHIN.			Cell volume.
		Whole blood.	Plasma.	Cells.	Whole blood.	Plasma.	Cells.	
1	32	160·0	140·0	206·6	8·0	4·8	15·44	30·0
2	40	173·2	182·0	158·6	11·7	6·9	20·4	35·5
3	18	150·0	131·2	227·6	..	4·5	..	19·5
4	40	148·0	140·0	173·3	10·6	4·3	30·55	24·0
5	42	160·0	..	..	11·1	9·9	12·9	39·0
6	56	160·0	..	..	..	..	..	35·8
7	25	130·0	..	..	7·2	..	..	20·7
8	18	154·0	..	..	10·3	..	..	20·1
9	30	138·0	..	..	10·2	..	..	28·0
10	28	135·5	..	..	..	..	..	35·2
11	25	160·0	..	..	11·3	..	..	25·6
12	30	160·0	..	..	10·0	9·2	11·86	30·5
13	30	154·0	..	..	12·0	..	..	30·3
14	9	107·5	..	..	7·0	..	..	20·6
15	15	142·8	..	..	..	..	..	29·4
16	23	182·0	..	..	9·6	..	..	26·4
AVERAGE	..	150·9	148·2	191·5	9·9	6·7	18·2	28·16

TABLE—*contd.*

(b) Chronic and recurrent cases.

Case number.	Age.	CHOLESTEROL.			LECITHIN.			
		Whole blood.	Plasma.	Cells.	Whole blood.	Plasma.	Cells.	Cell volume.
1	..	206·0	220·0	180·0	9·5	8·0	12·3	35·0
2	55	250·0	222·0	392·1	..	8·5	..	16·5
3	30	205·0	143·0	392·6	12·5	5·8	29·5	24·0
4	5	224·0	160·0	385·5	7·9	5·4	14·4	28·0
5	6	210·5	138·0	371·9	12·3	12·8	11·2	31·0
6	25	210·0	164·0	443·0	8·5	6·4	19·2	16·5
7	..	222·2	232·5	165·0	9·3	9·0	10·98	15·26
8	30	213·0	215·0	209·5	10·4	7·0	16·44	36·0
9	..	203·0	197·0	210·4	9·1	7·0	11·67	45·0
10	27	219·0	181·0	296·15	11·6	10·8	13·2	33·0
11	42	240·0	242·0	234·6	10·5	9·4	13·47	27·0
12	11	186·0	..	..	7·6	..	..	37·0
13	42	190·0	..	..	10·7	..	..	30·0
14	30	250·0	..	..	12·0	..	..	30·0
15	30	184·0	..	..	8·0	..	..	30·0
AVERAGE	..	216·8	192·2	298·4	9·8	8·2	15·2	28·95

TABLE—*contd.*

(c) *Cases with marked erythema legs.*

Case number.	Age.	CHOLESTEROL.			LECITHIN.			
		Whole blood.	Plasma.	Cells.	Whole blood.	Plasma.	Cells.	Cell volume.
1	25	210·0	164·0	442·7	8·5	6·4	19·12	16·5
2	16	205·0	194·0	245·16	10·0	6·6	22·4	21·5
3	..	186·0	200·0	144·0	10·0	7·2	18·4	25·0
4	42	240·0	242·0	234·6	10·5	9·4	13·47	27·0
5	38	..	250·0	266·6	10·0	6·0	..	..
6	40	333·0	..	..	11·0	..	..	20·0
AVERAGE	..	234·8	210·0	266·6	10·6	7·1	18·34	22·0

(d) *Cases with sarcoids.*

Case number.	Age.	CHOLESTEROL.			LECITHIN.			
		Whole blood.	Plasma.	Cells.	Whole blood.	Plasma.	Cells.	Cell volume.
1	25	210·0	164	242·7	8·5	6·4	19·12	16·5
2	30	228·6	190	338·4	9·0	6·0	17·5	26·0
AVERAGE	..	219·3	177	290·5	8·7	6·2	18·7	21·25

TABLE—*concld.*

(e) Cases with nutritional œdema.

Case number.	Age.	CHOLESTEROL.			LECITHIN.			Cell volume.
		Whole blood.	Plasma.	Cells.	Whole blood.	Plasma.	Cells.	
1	60	115·0	111·1	124·5	6·6	1·4	18·45	30·5
2	13	113·3	111·1	225·1	6·9	5·2	13·9	19·5
3	26	125·0	..	..	7·5	..	..	26·4
AVERAGE	..	126·6	111·1	174·8	7·0	3·3	16·1	25·5

(f) Cases of sub-acute glomerular nephritis.

Case number.	Age.	CHOLESTEROL.			LECITHIN.			Cell volume.
		Whole blood.	Plasma.	Cells.	Whole blood.	Plasma.	Cells.	
1	..	364	..	..	9·0	..	..	26
2	11	250	192·3	653·9	12·9	9·2	38·8	12·5

On referring to the Table we find that in acute cases the whole-blood cholesterol varies from 130 mg. to 182 mg. per 100 c.c. with an average of 150·9 mg. These figures fall within the normal range for Indians as determined by Boyd and Roy (*loc. cit.*) but the average is somewhat higher (116 mg. per 100 c.c. being the normal average for Indians). In chronic and recurrent cases the whole-blood cholesterol figures are decidedly higher than normal. They vary from 184 mg. to 256 mg. per 100 c.c. with an average of 216·8 mg. per 100 c.c. Cases with erythema and also those having sarcoids also show similar higher cholesterol contents. The figures for sub-acute nephritis are still higher, quite *normal values* however being obtained in œdema due to nutritional factors.

The average plasma cholesterol figures are of the same order as the whole-blood cholesterol in each group, but the cholesterol content of the cells is markedly higher

due probably to the lower cell volume, so that in the majority of cases there is distinct evidence of the greater concentration of cholesterol in the corpuscles.

The lipoid-P content of the whole blood in acute cases varies from 7 mg. to 12 mg. per 100 c.c. with an average of 9·9 mg. ; in recurrent and chronic cases the range is about the same (average 9·8 mg.) ; in cases having marked erythema variation is from 8·5 mg. to 11·0 mg. (average 10·6 mg.). Somewhat lower values, however, are obtained in cases having sarcoids (8·5 mg. to 9 mg. ; average 8·7 mg.). In few cases of nutritional œdema, the lipoid-P figures appear to be considerably reduced (6·6 mg. to 7·5 mg. ; average 7·0 mg. per 100 c.c.). Chopra and Roy (unpublished) determined lipoid-P in whole blood and also its distribution between the cell and plasma in normal Indians. The lipoid-P of the whole blood was found to vary between 9·8 mg. and 11·9 mg. (average 9·9 mg.) and the average lipoid-P content of the cell being 14·2 mg. per 100 c.c. (variation 13·1 mg. to 15·9 mg. per 100 c.c.). These figures are somewhat lower than the values obtained with respect to Europeans and Americans (12 mg. to 14 mg. per 100 c.c. of whole blood). Judging from Indian standards, the average lipoid-P values for whole blood fall within the normal range except those having sarcoids and suffering from nutritional œdema where lower values are obtained. The average lecithin contents of the plasma and of the erythrocytes do not show any marked deviation from the normal in the epidemic-dropsy cases, except in those showing marked erythema and sarcoids. Such cases show a somewhat greater concentration of lecithin in the cells than the majority of cases. In patients suffering from nutritional œdema a markedly lower value for plasma lecithin is shown without a corresponding increase in the lecithin content of the cells.

It is well known that in most cases of plasma-protein depletion the blood cholesterol is raised. This is probably a compensatory mechanism to increase the colloid-osmotic pressure. In epidemic dropsy though the plasma protein as a whole does not suffer any appreciable depletion, the albumin content which is considered mainly responsible for the regulation of the colloid-osmotic pressure is markedly reduced. The increase of blood cholesterol in this condition may well be explained as an attempt to adjust the lower colloid-osmotic pressure of the blood.

Moreover, the hydræmia which is a common feature of this disease may not be real as in hydræmic plethora, where there is an actual increase of blood volume. The hydræmia as is observed in this condition may only be relative owing to the smaller number of erythrocytes available in the circulation. With the disappearance of œdema and improvement of general condition there is a rise in the cell volume as well as in hæmoglobin and adjustment of serum proteins also takes place. Such an increase evidently is not due to regeneration of these elements as often the improvement is too rapid for regenerative processes to have been concerned. Moreover in stagnant capillaries, the corpuscles tend to adhere to the vessel walls and accumulate in them. In a healthy individual such accumulation may occur in the spleen, in the liver, and in bone-marrow, which might be designated as 'parking grounds' for the corpuscles. In epidemic dropsy where there is extensive and elaborate capillary dilatation such repositories for accumulation of red blood cells are abundant and widespread and therefore it is quite conceivable that a lesser

number of corpuscles become available for general circulation leading to a relative hydræmic condition of the blood.

SUMMARY AND CONCLUSIONS.

1.   The lecithin and cholesterol content of the blood of a number of persons suffering from epidemic dropsy has been determined.

2.   In acute cases the cholesterol content was found to be within the range for normal Indians but the average was somewhat higher.

3.   In chronic and recurrent cases there was a marked increase of the cholesterol content.   Similar higher figures were also obtained in those having sarcoids.

4.   The average lipoid-phosphorus figures showed normal values in both acute and chronic cases with or without erythema but somewhat lower values were obtained in those having sarcoids.

5.   In epidemic-dropsy lipoid-phosphorus figures vary within a much wider range than what is obtained in normal individuals.

6.   It is suggested that the increased cholesterol values may be a compensatory mechanism to increase the colloid-osmotic pressure of the blood which is markedly lowered in epidemic dropsy due to a depletion of the albumin fraction.

ACKNOWLEDGMENT.

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THE EFFECTS OF THE ORAL ADMINISTRATION OF ARGEMONE OIL TO LABORATORY ANIMALS.

BY

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CHOPRA and his co-workers (1939) showed that the oral administration of argemone oil (oil expressed from the seeds of *Argemone mexicana*) produces symptoms resembling those of epidemic dropsy. Five individuals who ate food cooked in mustard oil containing known quantities of argemone oil developed well-marked œdema and two of them showed the characteristic flushed appearance of the skin and had cardiac involvement (dilatation and murmur). It was also shown that argemone oil was more toxic to laboratory animals than mustard oil and that heating the adulterated oil at a temperature of 240°C. for 15 minutes led to the almost complete destruction of the toxic fraction in the oil. In this paper are recorded the results of further animal experiments with argemone oil. The oil was given by mouth to guinea-pigs with a bent cannula attached to a syringe. Mice were given the oil with a 2-inch hypodermic needle the end of which had been thickened and rounded off.

The results of the feeding experiments are given in tabular form. In Table I is the mortality in guinea-pigs fed with different doses of unheated and heated argemone oil and the mortality in control series of guinea-pigs fed with unheated and heated mustard oil, olive oil, and liquid paraffin. Six animals were used for

each dose. There was extreme loss of weight particularly in animals receiving argemone oil. In guinea-pigs which survived sub-lethal doses of argemone oil losses of 50 per cent or even more in body-weight were not uncommon and recovery required many weeks or even months.

<center>TABLE I.</center>

The mortality after the administration by mouth of different oils to guinea-pigs. Six animals were used at each dose.

Oil.	Treatment of oil.	Amount given each day, c.c.	Number of animals that died and day of death (average).	Average amount of oil (in c.c. per 100 grammes body-weight) that produced a fatal issue.
Argemone	Unheated	0·25	4 (30 days)	1·7
	,,	0·5	5 (12 ,,)	1·8
	,,	2·0	6 (6 ,,)	1·3
	Heated at 100°C.*	2·0	6 (5 ,,)	1·1
	,, ,, 170°C.	2·0	5 (9 ,,)	1·5
	,, ,, 240°C.	2·0	6 (17 ,,)	6·8
Mustard ..	Unheated	0·25	*Nil*	..
	,,	0·5	*Nil*	..
	,,	2·0	6 (12 days)	5·8
	Heated at 240°C.	2·0	4 (22 ,,)	14·0
Olive ..	Unheated	0·25	*Nil*	..
	,,	0·5	*Nil*	..
	,,	2·0	6 (13 days)	4·1
	Heated at 240°C.	2·0	6 (19 ,,)	8·8
Liquid paraffin.	Unheated	2·0	6 (30 days)	7·1

* The heating of the oil was carried out for 15 minutes at the stated temperature in all cases.

The feeding with oil was stopped in animals which appeared very ill. All animals received the standard stock laboratory diet and were looked after carefully.

There is an appreciable difference in the amount of the heated (at 240°C.) and the unheated argemone oil, mustard oil or olive oil that leads to the death of a guinea-pig. The difference is most marked with argemone oil, particularly when the 2-c.c. daily doses of heated (at 240°C.) and unheated oils are compared. The

lethal dose of unheated oil (or of oil heated at 100°C. or 170°C.) is between 1·1 c.c. and 1·5 c.c. per 100 grammes of body-weight, whereas 6·8 c.c. per 100 grammes body-weight of oil heated at 240°C. is required to kill the animal. This difference is significant.

Similar experiments were carried out on white mice. The results are recorded in Table II :—

TABLE II.

The effect of different daily doses of unheated argemone oil on white mice. Eight mice were used at each dose.

Daily dose (by mouth), c.c.	Average number of days animals survived.	Amount required to kill a mouse (in c.c. per 100 grammes body-weight).
Argemone oil (unheated).		
0·1	40	19·8
0·25	10	14·4
0·5	5	12·4
1·0	2·5	10·2
Olive oil 1·0	12	51·0

The experiment was repeated together with series of mice which received similar doses of the heated oil. The results are given in Table III :—

TABLE III.

The effect of different daily doses of unheated argemone oil and argemone oil heated at stated temperatures. Eight mice were used at each dose.

Daily dose (by mouth), c.c.	UNHEATED ARGEMONE OIL.		HEATED ARGEMONE OIL.		
	Average number of days of survival.	Amount required to kill a mouse (in c.c. per 100 grammes body-weight).	Temperature at which oil was heated.	Average number of days of survival.	Amount required to kill a mouse (in c.c. per 100 grammes body-weight).
0·5	5·6	13·2	100°C. for 1 hour	5	11·8
0·1	38	19·0	170°C. for 10 mins.	39	20·7
0·5	4·8	11·8	200°C. for 15 ,,	6	15·0
0·25	11	14·8	240°C. ,, ,,	14	17·4
1·0	2	10·0	250°C. ,, ,,	5·2	20·8

The effect of heating argemone oil is to make it less toxic to laboratory animals. This effect is most marked when the oil is heated at 250°C. for 15 minutes, i.e., when it ' fumes ' well. This is well seen in Table III which also confirms the results obtained in Table II. Argemone oil heated at 240°C. for prolonged periods still gave a well-marked positive reaction with nitric acid.

The tissues of animals that died during the experiment or that were killed when the animal was obviously ill were fixed in formaldehyde or Zenker's fluid, cut in paraffin and stained with hæmatoxylin and eosin. The tissues of animals that died during the night were not examined. The liver, kidney, stomach, and intestine from 10 mice and 2 guinea-pigs that had been given argemone oil were studied. The tissues of animals that had been given pure mustard oil or water only were also examined. The tissues in the control group showed no marked pathological changes.

The tissues from animals that had received argemone oil showed well-marked pathological changes. The organs showing extensive damage were the liver and the kidney. The changes were observed both in guinea-pigs and mice. The mucous membrane of the stomach and intestines was œdematous, the epithelial cells were swollen and had undergone cloudy changes and desquamation. The vessels of the villi were dilated. The liver showed portal congestion and thrombosis of some of the tributaries of the portal vein. There was marked fatty degeneration of the hepatic cells scattered throughout the lobules with small foci of necrosis near the periphery. No coagulated bile was seen in the hepatic ducts.

The kidneys presented a picture of acute hæmorrhagic glomerulo-tubular nephritis. There were well-marked degenerative changes in the convoluted tubules and descending limbs of Henle. The epithelium was detached in several areas in the form of tube casts. There was also marked intestinal congestion with extravasation and hæmorrhages in some areas.

The histological changes noted in the liver and kidneys are similar to those described by Kesten *et al.* (1939) in animal experiments with diethylene dioxide (dioxane). The changes in the liver in animals fed with argemone oil differ from those found in dioxane poisoning in that definite groups of hepatic cords drained by the tributaries of the hepatic veins are not affected. The vascular changes in the kidneys occur both in the cortex and in the medulla in animals fed with argemone oil whereas with dioxane the kidneys showed hydropic degeneration of the cortical region only. The changes produced by argemone oil are more extensive than those observed with dioxane. It is hoped to extend this work and to include the changes produced by different fractions (saponifiable and non-saponifiable) of the oil. Results of certain preliminary experiments carried out with these fractions suggest that the fatty acids of argemone oil are more toxic than the whole oil.

The amount of argemone oil required to produce symptoms in guinea-pigs and men was calculated. In animals a loss of more than 10 per cent of the original weight with loss of activity, and roughening of the coat were taken as indicating toxic effects. In man, the onset of aches and pains in the body and the development

of œdema were considered as indicating toxic effects. The amount necessary to produce these effects is shown in Table IV :—

TABLE IV.

Amount of argemone oil required to produce toxic effects in guinea-pigs and man.

Argemone oil.	Amount of argemone oil (in c.c. per 100 grammes body-weight) required to produce toxic effects.
(A) Guinea-pigs :—	
Unheated oil	0·54
Heated at 170°C. ..	0·95
„ „ 240°C. ..	1·23\
(B) Man :—	
Argemone oil, mixed in mustard oil used for cooking the food.	Approximately 0·88.

SUMMARY.

Argemone oil (the oil expressed from the seeds of the plant *Argemone mexicana*) is more toxic to laboratory animals than mustard oil, olive oil, or liquid paraffin. When the argemone oil is heated to a stage at which it 'fumes' well (at 240°C. for 15 minutes) the toxicity of the oil is not much more than that of certain bland oils, such as olive oil or liquid paraffin. The heated oil still gives a positive nitric acid test. Heating the oil at 100°C. or 150°C. has no appreciable effect in its toxicity to animals.

If the results obtained in animals can be confirmed by observations in man then the heating of the oil before use would be a simple method of controlling the outbreaks of epidemic dropsy.

Argemone oil when given by mouth to guinea-pigs or mice causes extensive degenerative changes in the liver and the kidneys. These changes have been described.

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ON THE ESTIMATION OF LACTOSE IN MILK.

BY

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In course of our investigation on the detection of diluted buffalo milk sold as cow milk satisfying the minimum standards for fat and non-fatty solids for cow milk, we found it necessary to adopt a rapid method for the estimation of lactose in milk. In the method described by Stewart and Boyd (1928), which has long been used in this laboratory, alumina cream is used to obtain a clear filtrate from milk and the quantity of lactose in it is estimated by the reduction of a known volume of Fehling's solution, using acidified potassium ferrocyanide as outside indicator for titration. The slow filtration after the precipitation of milk protein by alumina cream, repeated washing of the precipitate and the dilution to a definite volume at the end of thorough washing, make this method inconvenient for carrying out a number of lactose estimations in a short time. The use of an outside indicator is disadvantageous as is the tendency of the reduced copper to back oxidation whilst the outside test is being carried out. Hurst (quoted by Mitchell, 1930) used dialysed iron to obtain quickly a milk filtrate suitable for lactose estimation. We have found that this method in which dialysed iron alone is used, requires a large quantity of the reagent and does not work satisfactorily with fresh samples of milk, especially with those containing a comparatively high amount of lactalbumin and lacto-globulin.

Here we have described a method for obtaining, very quickly, a clear filtrate from milk by using acetic acid and a small quantity of dialysed iron. This filtrate is conveniently and accurately titrated against Fehling's solution using methylene blue as internal indicator. Some observations have also been made, regarding the colorimetric and polarimetric estimation of lactose in milk.

(953)

ESTIMATION OF LACTOSE ADDED TO A MIXTURE OF DIALYSED IRON AND CASEIN.

The following experiment was undertaken to determine whether the use of dialysed iron, for removing casein from milk, interferes with lactose estimation either due to adsorption or any other factor.

Experiment.

Casein (Hammerstan) was dissolved in sodium carbonate solution and neutralized to litmus with acetic acid. To 15 c.c. of 2 per cent casein solution was added 20 c.c. of bacto-lactose solution containing 0·4934 g. lactose (monohydrate) as estimated by Lane and Eynon's standard method (Sutton, 1935). The mixture was treated with 5 c.c. 1 per cent acid solution, 5 c.c. dialysed iron (B. D. H.) and shaken well. The volume was·made up to 100 c.c. and filtered. The lactose in the filtrate was determined by the same method mentioned above. The filtrate was found to contain 0·4955 g. lactose (monohydrate) per 100 c.c. The error, $+$ 0·0021 g. in the recovery of lactose is due to the fact that the 5 c.c. of dialysed iron which was added, contained some iron in the solid phase which did not act as a solvent for lactose. Consequently the concentration of lactose in the filtrate was a little higher. In repeated experiments the error remained constant, the filtration, however, was so quick and efficient that it was decided to adopt this method for the routine examination of lactose in milk.

A RAPID METHOD FOR THE ESTIMATION OF LACTOSE IN MILK.

Preparation of the milk filtrate.

Ten c.c. of milk* are pipetted into a measuring flask (100 c.c.) provided with glass-stopper. The pipette is washed well into the flask, with distilled water.

To this diluted milk, the volume of which is about 30 c.c. to 35 c.c., add 5 c.c. of 1 per cent acetic acid and shake the flask. Dialysed iron 5 c.c. is then gradually added, with rotation of the flask. The contents of the flask are vigorously shaken for about 2 minutes and then allowed to stand. When the precipitate has settled down, showing a clear supernatant fluid, the mixture is diluted to 100 c.c. and filtered. The filtration is very quick.

Titration of the milk filtrate.

The titration is performed on 10 c.c. Fehling's solution.† For accurate work duplicate titrations are carried out.

---

* For rapid work, the weighing of milk has been omitted. The specific gravity is determined and utilized in calculation.

† Soxhlet's modification of original Fehling's is used : (*a*) An aqueous solution of pure copper sulphate (Analar quality) containing 69·28 g. $CuSO_4$, $5H_2O$ per litre. (*b*) An aqueous solution containing 346 g. Rochelle salt and 100 g. of sodium hydroxide per litre.

(1) Preliminary titration (for ordinary routine work, this alone is sufficient). Milk filtrate 11·5 c.c. is added to 10 c.c. Fehling's solution and the contents are brought to boiling, addition of the filtrate is continued from the burette until there is left only a faint bluish tinge of the copper. Methylene blue (medicinal) 1 per cent aqueous solution, 3 drops are added and titration is finished when the liquid shows a bright orange colour due to the presence of cuprous oxide only. Let V be the volume of the filtrate required.

(2) Final titration (for very accurate work). Filtrate (V — 0·5) c.c. is added to 10 c.c. Fehling's solution and boiled for exactly 2 minutes from the time the mixture begins to boil briskly. Methylene-blue solution, 3 drops, are added and the titration finished in 1 to 2 minutes. Usually 0·1 c.c. to 0·5 c.c. more of the filtrate is required in this titration. Let V be the quantity of the filtrate required.

10 c.c. Fehling's solution = 0·0679 g. lactose (monohydrate) $C_{12} H_{22} O_{11}, H_2O$.

Calculation :—

$$\text{Per cent lactose in milk} = \frac{0.0679 \times 100}{V} \times \frac{100}{10 \times \text{sp. gr.}} = \frac{679}{V \times \text{sp. gr.} \times 10}.$$

Experimental.

The lactose content of a sample of milk was estimated by the rapid method described above using acetic acid and dialysed iron, and also by the method described by Stewart and Boyd using alumina cream. Methylene blue was used as indicator during Fehling's titration in both cases.

TABLE I.

Comparative estimation of lactose in milk filtrates prepared with (1) alumina cream and (2) dialysed iron.

Description of milk.	LACTOSE PER CENT.	
	Alumina cream.	Dialysed iron.
Cow No. 'L' ..	4·94	4·93
Silchar cow (adulterated sample) ..	2·71	2·77

Known quantities of lactose from standardized bacto-lactose solution, were added to samples of milk already analysed and estimation carried by the rapid method. Lactose was also estimated in milk diluted with known quantities of water. In every instance, 10 c.c. of the milk or mixture to be analysed was weighed in a chemical balance.

<div align="center">TABLE II.</div>

Estimation of lactose added to milk and in samples diluted with water.

Substance analysed.	LACTOSE PER CENT.		
	Present.	Found by analysis.	Per cent error.
Cow milk No. 'L'	..	4·79	..
„ + 1 per cent lactose ..	5·79	5·81	+0·02
„ + 0·5 „ „ ..	5·29	5·31	+0·02
„ + 0·25 „ „ ..	5·04	5·07	+0·03
Shillong buffalo milk (pooled) ..	..	4·3	..
„ „ milk diluted; 2 volumes + 1 volume water.	2·86	2·88	+0·02
Shillong farm cow (No. 70) milk	..	4·76	..
„ „ milk diluted; 1 volume milk + 1 volume water.	2·38	2·41	+0·03

Lactose content of a sample of formalin preserved milk was estimated by the rapid method at different dates.

<div align="center">TABLE III.</div>

Estimation of lactose in a sample of formalin preserved milk.

Description of milk.	Date of examination.	Lactose per cent.	REMARKS.
Cow No. 'L'	17–11–38	4·93	Fresh sample of milk examined and 3 drops (0·15 c.c.) formalin (40 per cent by volume) added to 6 oz. milk.
	21–11–38	4·89	
	25–11–38	4·89	
	29–11–38	4·92	

Table III, besides proving the reliability of the method of analysis, shows that the quantity of formalin added preserved the milk for 12 days without any significant deterioration to the lactose content.

Analyses of some representative samples of cow and buffalo milks by this rapid method are shown below.

TABLE IV.

Lactose content of cow and buffalo milks.

Description of milk.		Animal.	Lactose per cent.
18-4-39	Silchar No. 1	Buffalo	4·22
	,, No. 2	,,	4·95
	,, No. 3	,,	4·47
	,, No. 4	,,	4·91
	,, No. 5	,,	4·95
	,, No. 6	,,	4·94
29-4-39	Sylhet No. 11	,,	4·67
	,, No. 12	,,	4·16
25-5-39	Silchar No. 12	,,	5·11
29-5-39	Sylhet No. 6	,,	4·9
4-4-39	,, No. 123 ..	Cow	4·33
	,, No. 130 ..	,,	4·13
6-4-39	,, No. 116 ..	,,	4·73
12-4-39	Shillong farm No. 73 ..	,,	4·75
25-4-39	,, ,, No. 120 ..	,,	4·77
	,, ,, No. 58 ..	,,	4·22
	,, ,, No. 99 ..	,,	4·2
29-4-39	Sylhet farm No. 94 ..	,,	4·18
23-5-39	Shillong farm No. 123 ..	,,	4·8
9-6-39	Sylhet farm No. 30 ..	,,	4·94
16-6-39	,, ,, No. 26 ..	,,	4·02

COLORIMETRIC ESTIMATION OF LACTOSE.

Folin and Wu's method (Beaumont and Dodds, 1934) for the colorimetric estimation of blood sugar was used to estimate the lactose content of the milk filtrate. It was found that dialysed iron filtrate from milk proved as good as the protein-free filtrate obtained by 10 per cent sodium tungstate and $\frac{2}{3}$ N H_2SO_4, which is generally used in colorimetry.

In subsequent experiments on colorimetric estimation, the same dialysed iron filtrate, which was obtained from milk for Fehling's titration, was used after diluting

5 c.c. of the filtrate to 100 c.c. The method followed was essentially the same as in the estimation of blood sugar except that for copper reduction 10 minutes were allowed instead of 6 minutes during boiling. This modification was found necessary for thorough reduction of the copper by lactose. Standard lactose solution was prepared by diluting 4 c.c. of 0·5 per cent lactose in 0·25 per cent benzoic acid (standardized by Fehling's titration) to 100 c.c. 1 c.c. = 0·2 mg. lactose (standard solution).

Comparison was made in the colorimeter setting the standard at 20 mm.

R = reading of the (unknown) milk filtrate.

Calculation :—

$$\text{Lactose per cent} = \frac{80}{R \times \text{sp. gr.}} \cdot$$

Experimental.

TABLE V.

Colorimetric estimation of lactose by using (1) sodium tungstate and (2) dialysed iron filtrates of the same milk.

Description of milk.	LACTOSE PER CENT.	
	Sodium tungstate.	Dialysed iron.
Sylhet cow No. 12 ..	4·09	4·07 .

TABLE VI.

Colorimetric estimation of lactose and comparison with Fehling's titration method.

Number.	Description of milk.	LACTOSE PER CENT.						Difference.
		FEHLING'S TITRATION.			COLORIMETRIC.			
		Expected.	Found.	Error.	Expected.	Found.	Error.	
1	Shillong 'pooled' buffalo milk.	..	4·3	..	..	4·08	..	— 0·22
2	Buffalo milk 2 volumes + w a t e r 1 volume.	2·88	2·88	*Nil*	2·72	2·64	— 0·08·	— 0·24

TABLE VI—*concld.*

Number	Description of milk.	LACTOSE PER CENT.						Difference.
		FEHLING'S TITRATION.			COLORIMETRIC.			
		Expected.	Found.	Error.	Expected.	Found.	Error.	
3	Shillong farm cow No. 101.	..	4·92	..	..	5	..	+ 0·08
4	Cow milk 3 volumes + water 1 volume.	3·69	3·7	+ 0·01	3·75	3·6	− 0·15	− 0·1

When the blue colour developed is compared and readings taken in a colorimeter of the type (Klett-Bio) we have used, it has been our experience that there is always a chance of error up to ± 1 in the readings which corresponds to ± 0·2 per cent lactose (when the standard is set at 20 mm.). This explains the difference between the values of lactose obtained by the colorimetric and the Fehling's titration method.

POLARIMETRIC METHOD OF LACTOSE ESTIMATION.

The varying amounts of precipitate produced during the preparation of a clear filtrate from different samples of milk cause considerable difficulty in the accurate estimation of lactose in milk by polarimetric method. Wiley's method (quoted by Blyth and Blyth, 1909), which suggests a correction to include fat and protein, is not suitable for routine work.

Liverseege (1932) describes Vieth's method modified by Richmond and Boseley. Following this method a clear filtrate suitable for polarization was obtained by adding 3 c.c. mercuric nitrate solution* to 100 c.c. milk. Polarization was carried out in 200-mm. tube in a Franz Schmidt and Haench type polarimeter.

The correction applied was, as shown below :—

$$\text{Anhydrous lactose per cent} = \frac{100 \, a - (1\cdot075 \times F \times S)}{110\cdot6 \, S}$$

Where a = degrees of rotation

F = percentage of fat

S = sp. gr. of milk

Lactose (monohydrate) = anhydrous lactose × 1·053.

* Mercuric nitrate solution is prepared by dissolving 5 c.c. mercury in 96 c.c. concentrated nitric acid and diluting the product with an equal volume of water.

Experimental.

A few samples of milk were analysed for lactose content by the Fehling's titration (using acetic acid and dialysed iron for obtaining the filtrate) and by the polarimetric method. The values for lactose together with specific gravity, fat, and non-fatty solid are shown in Table VII. Fat was estimated by Gerber's method, specific gravity determined with lactometer, and total solids were weighed as usual after evaporation to dryness.

TABLE VII.

Detailed analysis of milk including polarimetric estimation of lactose.

Date of analysis.	Source of milk.	Specific gravity.	Fat.	Non-fatty solids.	Lactose per cent.		
					Fehling's titration (rapid method).	Polari-metric.	Difference.
20-1-39	Shillong farm cow No. 129	1034	5·3	10·2	4·99	5·37	+ 0·38
	,, ,, ,, No. 140	1034	2·9	9·1	4·81	5·21	+ 0·4
23-1-39	Sylhet farm cow No. 140	1032	2·5	8·7	4·62	4·86	+ 0·24
	,, ,, ,, No. 30	1035	7·2	10·8	4·31	4·8	+ 0·49
	,, ,, ,, No. 6	1035	3	9·2	4·89	5·39	+ 0·5
	,, ,, ,, No. 9	1035	5	9	4·7	4·98	+ 0·28
24-1-39	,, ,, ,, No. 96	1030	8·2	8·1	4·09	4·58	+ 0·49
	,, ,, ,, No. 108	1032	7·2	8·5	4·91	5·37	+ 0·46
	,, ,, ,, No. 130	1035	3·9	9·2	4·88	5·2	+ 0·32
27 1-39	Sylhet farm buffalo No. 12	1035	8·7	10·3	4·91	5·33	+ 0·43
31-1-39	Sylhet farm cow No. 62	1034	5	9·5	5·27	5·6	+ 0·37
	,, ,, ,, No. 56	1034	5·5	8·6	4·61	4·98	+ 0·37

The values of lactose obtained by polarimetric estimation are + 0·24 to + 0·5 per cent higher than those obtained by the Fehling's titration method.

DISCUSSION.

The method described here, in which acetic acid and dialysed iron are used for obtaining a clear milk filtrate and Fehling's titration method carried out using methylene blue as internal indicator, requires a very short time in comparison with other

known volumetric methods. This rapid method, however, shows an error of $+ 0.02$ to $+ 0.03$ per cent (*vide* Table II) on account of the dilution of 10 c.c. milk sample together with 5 c.c. dialysed iron, up to 100 c.c. as the first step in the analysis. The distribution of lactose is not homogeneous throughout, this 100 c.c. of the fluid containing some iron oxide. In the calculation, however, a homogeneous distribution is assumed and hence the error. On evaporating 5 c.c. dialysed iron to dryness it is found to contain 0.35 g. solids, hence not more than $(100 \text{ to } 0.35) = 99.65$ c.c. of fluid act as solvent for the lactose. If the observed percentage of lactose by this method be corrected as follows the plus error is neutralized or minimized to a great extent :—

$$P = P^1 \times 99.65$$

Where P^1 = observed percentage of lactose after titration of the dialysed iron milk filtrate.

P = actual per cent lactose content in the milk.

This method has been conveniently applied by us in the analysis of about 500 samples of milk from cow and buffalo of known breeds for the lactose content (the results of analysis, together with the history of animals and other observations will be published elsewhere).

It has already been explained that the colorimetric estimation of lactose by the method described before is liable to an error of ± 0.2 per cent. Further observation has revealed that the colour developed by the reduced copper is strictly proportional to the concentration of lactose only when the unknown solution contains almost the same quantity of lactose as the standard solution. When different samples are matched against a single standard, sometimes the error is more than ± 0.2 per cent.

The colorimetric method, though unsuitable for determining the exact percentage of lactose in a sample, will be found useful for furnishing additional evidence in routine analysis of a large number of milk samples, when certain minimum standards for lactose have been established for cow and buffalo milks.

The polarimetric method which we have followed gives higher percentage of lactose than that obtained by the copper reduction method.

SUMMARY.

A rapid volumetric method for the estimation of lactose in milk has been described and some observations made regarding the colorimetric and polarimetric methods. Lactose contents of a few representative samples of cow and buffalo milks have been determined.

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THE EFFECT OF WHEAT DIET IN THE RELIEF OF CERTAIN PAINFUL COMPLICATIONS AND SEQUELÆ IN LEPROSY.

BY

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THE importance of diet in the epidemiology and treatment of leprosy has been stressed by many workers since the publication of Jonathan Hutchinson's book on leprosy and fish-eating in the latter part of the 19th century, a book which stressed above all other ætiological factors the possibility of a certain type of food being a causative factor in the acquirement of leprosy. The lack of various nutritional factors has been blamed from time to time for the spread of leprosy in a community; but the consensus of opinion among leprologists is that, while diet-deficiency may play some part in the epidemiology of leprosy, this is of secondary importance in comparison with such factors as contact with open cases and age.

No conclusive investigations have yet been undertaken which indicate with any degree of certainty the additional dietary factors which may be of value in the treatment of leprosy. With the collaboration of Dr. W. R. Aykroyd (the Director of the Nutrition Research Laboratories, Coonoor) certain dietetic experiments have been conducted during the past two years in the Lady Willingdon Leper Settlement. We do not propose to present the findings of these investigations in general because the results are still inconclusive. This paper deals with one interesting observation made in the course of the work, namely, the good effect of a wheat diet in the relief of painful neuritis and certain distressing bone and joint pains.

Some years ago Dr. Flora Innes of the Vellore Medical College, when discussing certain dietetic questions with one of us (R. G. C.), expressed the view that excessive consumption of carbohydrates may give rise to various signs of metabolic imbalance, and suggested that wheat should be substituted for rice in the diet of

the institution. A review of the women patients in the Settlement showed that many were suffering from acute bone pain not infrequently accompanied by nerve pain. The latter symptom is common in leprosy, and leprous neuritis is a most distressing sequelæ of the disease. It is known that, when there is definite swelling and a tendency to necrosis, incising the sheath will relieve pain. Recently, Lowe (1939) has suggested the surgical removal of the nerve-sheath as a satisfactory operation for the relief of nerve pain, but this procedure has only limited application. In the Lady Willingdon Leper Settlement, the large tender swollen nerve with abscess formation is rare, but nerve pain, of a chronic persistent gnawing type, due partly to swelling of the nerve-sheath, but more often to fibrosis, is very common.

It was decided to try the effect of a wheat diet on patients with severe and chronic nerve and bone pain. The latter is not a sequela of leprosy, but a common complication; the bone pain is frequently so severe that the slightest pressure gives rise to the most excruciating pain.

RESULTS.

Thirty-seven cases in all have been observed. In every case in which the wheat diet was consumed for three months and more, there has been either complete relief of these distressing symptoms or they have been very considerably alleviated (*see* Table II). One of us (M. D. S.) has noticed a remarkable change in the temperament of the women who have been placed on wheat diet, and has observed that the consumption of anodynes, such as a powder containing aspirin, phenacetin and caffein, has been reduced to negligible proportions.

Table I shows the standard diet issued in the Settlement :—

TABLE I.

A. Standard children diet.			B. Indian diet (general).		
		Oz.		Lb.	Oz.
Parboiled rice	..	15·00	Rice parboiled ..	1	2
Onions ..	..	0·30	or		
Dhal (pulse)	..	1·50	Rice raw (with curd) ..	1	2
Ghee	..	0·40	,, (with mutton)	1	1
Mutton ..	..	2·40	Dhal (mutton days) ..	0	4
Vegetables	..	6·70	,, (other days) ..	0	6
Coco-nuts	..	No. 0·02 (4 coco-nuts for 214 children).	Vegetables ..	0	8
			Onions	0	0½
Plantains	..	1 (one)	Ghee	0	0½
			Mutton (Wednesday and Saturday)	0	4
			or		
			Curd (daily) ..	1 Ollock (approximately ¼ pint in volume).	

With condiments and spices in small quantities.

Note.—The above figures were obtained by dividing the average daily kitchen issue by the average number of children fed, i.e., 214.

With condiments and spices in small quantities.

Note.—Indian women receive 1 lb. 0 oz. of rice instead of 1 lb. 2 oz. Otherwise the issue is the same as for men.

TABLE I—*concld.*

C. Anglo-Indian diet (general).

(Central kitchen issue.)

	Lb.	Oz.
Coffee beans	0	$0\frac{1}{2}$
White sugar	0	$3\frac{1}{2}$
Milk	0	4
White bread	0	8
Eggs (one on three days, two on four days per week).		
Butter	0	1
Raw rice	0	6
Mutton (3 oz. on four days—6 oz. on three days per week).		
Ghee	0	$1\frac{1}{4}$
Onions	0	2
Potatoes	0	3
Vegetables (4 oz. on three days—8 oz. on four days per week).		
American flour	0	$0\frac{1}{4}$
Tea	0	$0\frac{1}{8}$
Coco-nuts	$\frac{1}{10}$ of a coco-nut per head.	

With condiments and spices in small quantities.

From the above diets all rice was eliminated and instead whole wheat was taken. This was lightly ground on the currystone and cooked in the same way as rice and distributed to the patients who ate it along with their usual curries. In the evening chapatties were eaten. For adults 21 oz. of wheat *per diem* were supplied and for boys 12 oz. Wheat prepared in this way needs slightly longer cooking than rice and extra salt should be added. None of the patients liked the wheat and it was with the greatest difficulty that they were persuaded to continue eating it, even though their symptoms had completely abated. So accustomed is the South Indian to rice that he would rather have pains than do without rice.

TABLE II.

Percentage of cases showing complete or partial relief of nerve and bone pain after the administration of the wheat diet.

	Total given wheat diet.	Complete relief.		*Partial relief.		PERCENTAGE.	
						Complete relief.	Partial relief.
		M.	F.	M.	F.		
(a) Bone pain ..	14	7	5	0	2	85·7	14·3
(b) Nerve pain ..	23	12	4	6	1	69·5	30·5
Total number of cases	37	Total completely relieved				75·7	

* Slight tenderness of nerves still present and occasional pain but not severe.

Throughout the experiment all patients continued to receive treatment for leprosy. The standard treatment employed in this institution is subcutaneous injections of hydnocarpus oil with 2 per cent creosote, and a mixture of ethyl-esters of hydnocarpus oil intradermally. The formula for the latter mixture is as follows: ethyl-esters of hydnocarpus oil 75 parts, olive oil 25 parts, and creosote four parts. The majority of the patients in this experiment were unable to tolerate dosages of hydnocarpus oil above 5 c.c. The administration of the wheat diet did not appear to increase their tolerance to the hydnocarpus preparations, nor was there any indication that there was any more rapid improvement in their leprosy (*see* Table III).

TABLE III.

Analysis of cases indicating the condition as regards leprosy after the administration of wheat.

Negative.	Much improved.	Improved.	Stationary.	Worse.	TOTAL.
2	2	18	14	1	37
(1·54 per cent)	..	(9 F.)	(3 F.)	..	..

SELECTED ILLUSTRATIVE CASES.

1. *Kanniammal, female, aged 40 years, type, L2.*—Placed on wheat diet November 1937. Complained two or three times a month of pains in knees and hands and long bones of extremities. Sometimes her eyes were red and painful. Occasionally she complained of difficulty in breathing. Liniments, ichthyol, and glycerine and antiphlogistine tried, but no permanent relief. Aspirin and

phenacetin brought only temporary relief. Two months after wheat diet commenced, joint and nerve pains were completely relieved. No further laryngeal attacks, and eye pain much improved. No local treatment given and no need for the administration of anodynes. The wheat diet experiment was discontinued in August. Two months later symptoms recurred and the patient is again in hospital.

2. *Allima Bee, female, aged 32 years, type L1.*—Placed on wheat diet November 1937. Patient was an unhappy discontented woman. For months at a time unable to do her own cooking and housework, because of weakness and joint and bone pains. Frequently found lying on her mat weeping. She continually complained of pain and had attacks of severe abdominal discomfort and sometimes pain ; at one time cholecystitis was suspected. Two months after taking wheat began to feel better, and indigestion was completely relieved. Joint and bone pains were less. After three or four months she could do her own cooking and housework, and appeared more cheerful and talked more to her neighbours. After six months, much stronger and seldom seen lying on her bed ; is now working as a ward ayah and is bright and happy.

3. *Amirtham, female, aged 35 years, type L1.*—Placed on wheat diet November 1937. Patient suffered from severe joint and bone pains. Grasping the ulna or tibia firmly and pinching the clavicle caused her excruciating pain. She was unable to do her housework for months at a time and was weak and unhappy ; frequently found crying. Local applications to painful joints only soothed for one or two hours, the administration of anodynes, such as aspirin and phenacetin, enabled the patient to get a little sleep. After taking wheat for two months the patient appeared stronger and happier ; by the end of three months all pains had completely disappeared and she has been able to do her own cooking and housework for the past year. She now seldom complains of pain and never asks for a powder. Has been working as an Anglo-Indian orderly for the past four months ; looks well and is happy.

4. *Ellammal, female, aged 38 years, type L2.*—Placed on wheat diet November 1937. Complained of joint and bone pains but these were never severe. Pains subsided after three to six months and she no longer complains.

5. *Arputhammal, female, aged 40 years, type L2.*—Patient was emaciated and weak with deformed hands and feet. Scarcely able to move about. Complained of nerve pains and pains all over the body, especially hands and knees. Local applications brought little relief. A powder containing aspirin, phenacetin and caffein, and a mixture of sodium salicylate gave only temporary relief. After three months noticed great improvement ; her health improved and she is more cheerful and stronger and is able to get about. Has not had severe pain for months and tenderness in ulna and peroneal nerves has completely subsided.

6. *Ekambaram, male, aged 18 years, type L2.*—Placed on wheat diet November 1937. Before taking the wheat diet patient was a thin, miserable-looking boy continually complaining of nerve pain, joint pain, and severe bone pain. The latter was especially noticeable over the tibia, radius, ulna, and clavicles, and considerable pain was caused on pressure. The patient's joints were frequently swollen ; he showed no desire to play with other boys and was always wanting to stay in hospital. Local applications brought little relief and the usual anodynes had to be frequently administered which gave some relief for a few hours. In addition to the usual diet supplied he was given 1 pint of milk and 1 egg per day, but in spite of this no improvement was noticed in his condition. In November 1937, after much pressure, he was persuaded to take the wheat diet. After about three months his symptoms began to abate and he started putting on weight and looked more cheerful. Six months after being placed on the wheat diet, he refused to continue, but on the recurrence of severe bone pain which had previously become much less severe, pleaded to be given wheat diet again. From this time onwards his improvement was steady and remarkable as shown by the following record of admission to hospital :—

27th December, 1935 to 28th November, 1937—Total days in hospital—521.

Placed on wheat diet—November 1937.

			Days.
December 1937 to July 1938	..	..	..	40
July 1938 to August 1939	..	..	..	30
September 1938 to May 1939	..	..	..	37
9th May, 1939 to August 1939	..	..	..	*Nil*

It is interesting to note that during the period this boy was off wheat diet (approximately 2 years ; this includes the one month July to August 1938, when he refused wheat diet) his total stay in hospital was 551 days. After being placed on wheat diet, he only spent 77 days in hospital in a period of 19 months (excluding the month July-August 1938, when he refused the wheat diet). Since May

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1939 has kept very well and is looking healthy. Now he is always seen playing with other boys. Recently has been doing duty as ward boy.

7. *Ramakrishnan, male, aged 16 years, type L2.*—Placed on wheat diet May 1938. Constantly in hospital with attacks of nerve pain with poor nutritional state of skin, showing impetiginous eruptions; nerve pains completely subsided and skin condition markedly improved. Patient now looking happy and playing as a normal child should play.

8. *Anglo-Indian, male, aged 39 years, type L2N2.*—Placed on wheat diet February 1939. This patient requested that he might be given wheat, because of acute tenderness in ulnars, right great auricular and peroneal nerves. The great auricular nerve in particular was enlarged, and very tender. After six weeks, improvement commenced, and all pains completely disappeared in three months, except for slight tenderness in right ulnar nerve. From February 1939 to August 1939 this patient was not in hospital, except when he was brought in for a circumcision to be performed, in October 1939. Two months after stopping wheat he has had a recurrence of acute and severe nerve pain, and was in hospital. The patient has again been placed on wheat diet as a therapeutic measure and one month later nerve pain had almost completely ceased.

9. *C. Vythalinga Padavachi, male, aged 42 years, type L1.*—Placed on wheat diet January 1939. Complained of frequent reactions and severe pain and tenderness of nerves with excruciating bone pain. Any pressure on the anterior border of the tibia, or any compression of the ulnars, or clavicle was so painful that tears came into the patient's eyes. Further, he walked with a stopping gait owing to the constant pain. After three months the severe bone pain had completely disappeared and firm pressure on the large bones of the arms and legs caused no pain, nor did pinching the clavicle elicit any tenderness. The patient walked with an upright gait and looked well, having lost the anxious look of a person suffering from persistent and continuous pain. The patient's nerve pain was almost completely relieved but there was still some tenderness of the nerve and occasional pain but not of a severe type. Two months after stopping the wheat diet this patient relapsed to his former distressing condition.

DISCUSSION.

This experiment was conducted for a sufficiently long period to eliminate any possible psychological factor. Further, this factor could hardly have played any part of importance as the majority of patients were reluctant both to commence the wheat diet and continue it when it was once started. The objection may be made that there were no controls, but in one sense the patients themselves were their own controls, for none were put on to the wheat diet who had not previously failed to respond to palliative treatment. The very fact that there has not been a single complete failure in this experiment is, we think, of some significance. One of us (M. D. S.) knew the condition of many of the patients intimately prior to the administration of wheat and can vouch for their remarkable improvement. Apart from the change in diet, there was no alteration in the living conditions of the patients.

It is interesting to note that whereas the improvement in nerve pain was constant and frequently remarkable, there was no corresponding improvement in the leprosy condition. In fact the percentage of negatives (5·4 per cent) was very much lower than the average in the institution (20 per cent). It is admitted that many cases were advanced, but even so one would have expected a more favourable result had the wheat diet had any effect on the leprotic condition. Without exception those patients on wheat diet showed a very marked improvement in their general condition. This indicates that caution is necessary in ascribing lack of improvement in leprosy to a dietetic factor or to a poor general state of health. It is often suggested, with too much facility, that these causes underlie deterioration in leprosy, whereas the real factor is often unknown and needs much more intensive study.

The original diets of the groups showed considerable variation. For instance, the diet of the Anglo-Indian group was very different from that of the Indian. Yet the Anglo-Indian patient who had this diet in addition to the wheat (the small quantity of rice alone being replaced by a larger issue of wheat) showed the same type of improvement. All patients in the experiment were non-vegetarians, and consumed, prior to being given the wheat diet, ' ordinary rice ', that is parboiled rice which is known to have a higher vitamin-B_1 content than ordinary raw rice. It, therefore, seems unlikely that vitamin B_1 was the main factor in causing improvement. Kiel (1938), Blueth (1939), and others have suggested that the administration of vitamin B_1 may prove of service in leprous neuralgia. We have been unable to confirm this observation and consider that the answer to the problem is not quite so simple. The improvement in our series of cases was probably due to a combination of food factors, including protein, calcium, and the vitamin-B complex. The relative importance of each has not yet been determined. It is further interesting to note that since the cessation of the experiment in August 1939 three cases relapsed to their former state; one of which, the Anglo-Indian, has again recovered after having recommenced the wheat diet.

CONCLUSIONS.

The series of experiments described in this paper indicate that the administration of a whole-wheat diet to patients with leprosy who complain of severe and chronic nerve and bone pain is of value and that it is worthy of a trial when other remedies have failed. It·is suggested that this should be tried as a therapeutic measure before deciding to operate for a painful neuritis, except in cases where there is marked swelling of the nerve-sheath and threatened abscess-formation.

The administration of a whole-wheat diet, in addition to the hydnocarpus preparations, appears to have no effect in hastening improvement in lepromatous cases, nor does it appear to increase the percentage of those who become negative; by negative is meant the disappearance of the *M. lepræ* from the skin and mucous membrane of the nose, standard methods of examination being employed.

ACKNOWLEDGMENTS.

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ACTION OF SULPHANILAMIDE DERIVATIVES IN *STREPTOCOCCAL* AND *PNEUMOCOCCAL* INFECTIONS IN MICE.

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THE synthesis and the pharmacological study of new derivatives of sulphanilamide are of twofold importance. First, they help in obtaining compounds which may be superior to sulphanilamide and 2-sulphanilamidopyridine (M. & B. 693) or prove effective in diseases in which these drugs fail. Secondly, they aid in understanding the fundamental issues as the mode of action and the relation of the chemical constitution to chemotherapeutic action of this class of drugs.

Two of the factors that limit the chemotherapeutic activity of sulphanilamide and hence require frequent repetition of the drug to keep up the optimum blood concentration are: (*i*) the conjugation of the drug in the body to yield the inactive acetyl derivative and (*ii*) the rapid excretion of the drug from the body. It is held by some workers that the therapeutic effect of sulphanilamide is due to its being oxidized to the hydroxylamino-, nitroso-, or nitro-derivative by the atmospheric oxygen under the catalytic influence of respiring tissues or organisms (Mayer, 1937, 1938; Schaffer, 1939). The object of the studies undertaken is to obtain suitable derivatives which, besides proving superior to sulphanilamide, may, in addition, serve (*i*) to elucidate the factors that govern the acetylation and the rate of excretion of the compounds and (*ii*) to get an insight into the mechanism of action of these drugs. In this paper are described the results of testing some compounds related to sulphanilamide in

streptococcal and *pneumococcal* infections in mice. In one class of compounds synthesized suitable substituents in the amino part of the molecule have been introduced, such that the amino radical can undergo neither acetylation nor oxidation. In the other class, the amido group of the molecule is substituted with different types of acid groupings to test how they influence the chemotherapeutic activity, the blood concentration, and the rate of excretion.

EXPERIMENTAL RESULTS.

Of the many compounds synthesized (Ganapathi, 1938*a, b*; 1939), seventeen were kindly tested by Dr. G. A. H. Buttle in mice infected with *β-hæmolytic streptococci* (Richard's strain) and *pneumococci* (type I).

(1) 3'-methoxy-4'-hydroxybenzylidene *p*-aminobenzenesulphonamide.

(2) 4-amino-5-[4'-sulphonamidophenylazo]uracil.

(3) 4-amino-5-[4'-sulphonamidophenylazo]thiouracil.

(4) 2-N^1-sulphanilamidobenzoic acid*.

(5) N-sulphanilyl-(β)-phenyl-(α)-alanine.

(6) *p*-[3'-methoxy-4'-hydroxybenzylamino]benzenesulphonamide.

(7) 4-nitro-4'-aminodiphenylsulphide.

(8) *p*-[*w*-allylthiocarbamido]benzenesulphonamide.

(9) 4 : 4'-di-[*w*-allylthiocarbamido]diphenylsulphone.

(10) Product obtained by coupling diazotized sulphanilamide with abietic acid [Fieser and Campbell, 1938].

(11) N-sulphanilyltaurine.

(12) 3-N^1-sulphanilamidocinnamic acid.

(13) 1-N^1-sulphanilamidonaphthalene 3 : 6-disulphonic acid.

(14) 2-N^1-sulphanilamidonaphthalene 5 : 7-disulphonic acid.

(15) 1-N^1-sulphanilamido 8-naphthol 3 : 6-disulphonic acid.

(16) 2-N^1-sulphanilamido 8-naphthol 3 : 6-disulphonic acid.

(17) 6-N^1-sulphanilamidoquinoline.

The experimental results with the compounds that showed considerable activity are presented below. In all these tests, sulphanilamide was taken as the standard in the *streptococcal* and 2-sulphanilamidopyridine (M. & B. 693) in the *pneumococcal* infections in mice.

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* The nomenclature adopted in this paper is that suggested by Crossley *et al.* (1938).

TABLE I.

Mice were inoculated with hæmolytic streptococci (*Richard's strain*) *intraperitoneally* (50,000 cocci).

The drugs were given by mouth immediately after infection and repeated 6 hours, 1, 2, 3, and 4 days later.

Compound tried (number refers to that in the list).	Number of mice dying out of a group of 6, on each day after infection.								Survivors 9 days.
	1	2	3	4	5	6	7	8	
Controls	5	1	..	..	..	..	..	..	0
Sulphanilamide : 10 mg. ..	0	0	0	0	0	1	0	1	4
No. 1 : 10 mg. ..	0	0	0	0	0	0	0	0	6
No. 2 : 10 mg. ..	0	0	0	0	0	3	0	0	3
No. 6 : 10 mg. ..	1	1	1	0	0	1	0	0	2

Toxicity.—The drugs were tolerated by mice in 200 mg. doses.

TABLE II.

Same as the previous test (hæmolytic streptococci).

Compound tried.	Number of mice dying out of a group of 6, on each day after infection.				Survivors 5 days.
	1	2	3	4	
Controls	4	2	..	..	0
Sulphanilamide : 1 mg. ..	0	1	0	1	4
No. 1 : 1 mg. ..	0	1	3	1	1
No. 2 : 1 mg. ..	0	2	2	2	0

TABLE III.

Mice inoculated with hæmolytic streptococci (*Richard's strain*) *intraperitoneally* (30,000 cocci).

The drugs were given immediately after infection and repeated 5, 24 and 48 hours later.

Compound tried.	Number of mice dying out of a group of 6, on each day after infection.					Survivors 6 days.
	1	2	4	5	6	
Controls ..	4	2	..	..	..	0
Sulphanilamide: 10 mg. ...	0	0	0	0	0	6
Sulphanilamide: 1 mg. ..	0	0	2	0	0	4
No. 4: 10 mg. ..	0	0	2	1	0	3
No. 4: 1 mg. ..	4	2	..	..	..	0
No. 5: 10 mg. ..	0	0	3	0	0	3
No. 7: 10 mg. ..	0	0	5	0	0	1

Toxicity.—The drugs were tolerated in 100 mg. doses (by mouth) by the mice.

TABLE IV.

Same as the previous test (hæmolytic streptococci).

Compound tried.	Number of mice out of a group of 6, dying on each day after infection.						Survivors 7 days.
	1	2	3	4	5	6	
Controls ..	6	..	..	..	..	..	0
Sulphanilamide: 10 mg. ..	0	1	0	1	1	0	3
Sulphanilamide: 1 mg. ..	0	4	0	1	1	0	0
No. 3: 10 mg. ..	0	2	1	2	0	0	1

Toxicity.—The drug was tolerated by mice in 100 mg. doses.

TABLE V.

Mice inoculated with pneumococci *(type I) intraperitoneally* (30,000 cocci).

The drugs were given immediately after infection and repeated after 6 hours, 1, 2, 3, and 4 days later.

Compound tried.	Number of mice out of a group of 6, dying on days after infection.										Survivors.
	1	2	3	4	5	6	7	8	9	10	
Controls	0	5	0	0	0	0	0	0	0	0	1
2-sulphanilamidopyridine (M. & B. 693) : 30 mg.	0	0	0	0	1	0	0	0	0	0	5
Sulphanilamide : 30 mg. ..	0	0	0	2	0	2	0	0	0	1	1
No. 1 : 30 mg. ..	0	0	1	2	0	1	0	0	0	0	1

2-N′-sulphanilamidothiazol, one of the compounds synthesized and tested in this Institute, showed a very striking action in *streptococcal* and *pneumococcal* infections in mice (detailed report to be shortly published). The compounds (1), (2), and (3) are very slightly inferior to sulphanilamide in *streptococcal* infections in mice. The compound (1) in 10 mg. doses is at least equal to sulphanilamide in activity. These compounds are far less toxic than sulphanilamide.

The compounds (4) and (5) are about half as active as sulphanilamide in *streptococcal* infections. In 1 mg. doses compound (5) possessed no protective power. Crossley *et al.* (*loc. cit.*) have reported, without giving details of their experiments, that the compound (5) is superior to sulphanilamide. The compounds (6) and (7) show only slight activity.

The compounds (8) to (16) showed very little or no activity in experimental *streptococcal* and *pneumococcal* infections when tested in 10 mg. and 30 mg. doses, respectively. The quinoline derivative (17) was extremely toxic, a single dose of 5 mg. and also 2·5 mg. killing one out of two mice in each experiment and only a dose of 1·25 mg. not proving fatal. In 0·5 mg. doses its therapeutic activity was practically *nil.*

The only compound of the series that showed striking activity in *pneumococcal* infections was 2-sulphanilamidothiazol which is as active as 2-sulphanilamido-pyridine (M. & B. 693). This indicates that for pronounced therapeutic action in *pneumococcal* infection, a pyridine or probably a heterocyclic ring in the sulphonamide part is essential.

DISCUSSION.

The common denominator for the striking antibacterial action of the aromatic sulphur compounds appears to be the grouping—N⟨◯⟩S—. The other structural or physico-chemical details which govern the degree of therapeutic activity have not been thoroughly elucidated though some empirical rules have been formulated within a narrow range of structural changes (Buttle *et al.*, 1938; Crossley *et al.*, *loc. cit.*; Marshall, 1939*a*, *b*; Rosenthal *et al.*, 1939; Trefouel *et al.*, 1937*a*). The results recorded here have brought to light some interesting points.

It has been shown by Trefouel *et al.* (1937*a*) that the modification of the amino group in sulphanilamide into a formyl, acetyl, or urethanyl derivative decreases the therapeutic activity to a great extent. The conversion of the amino group into a urea derivative destroys the activity, the symmetrical urea derivative obtained from sulphanilamide (1192 F.) being inactive. In accordance with this, the conversion of the amino group into the allylthiourea derivative completely destroys the activity—*p*-[*w*-allylthiocarbamido]benzenesulphonamide (8) and 4 : 4'-di-[*w*-allyl-thiocarbamido]diphenylsulphone (9) being inactive, although allylthiocarbamide (' thiosinamine ') itself is known to possess definite anti-*streptococcal* properties. This indicates that the accumulation of therapeutically active groups in the same compound does not increase the activity.

Gray, Buttle and Stephenson (1937) tested a series of Schiff's bases derived from sulphanilamide and found that their anti-*streptococcal* activity was about equal to that of sulphanilamide, some being a little more and some a little less active, but these compounds were uniformly far less toxic. To see whether the presence of the guaiacol residue will enhance the therapeutic activity of the anils, 4'-hydroxy-3'-methoxybenzylidene *p*-aminobenzenesulphonamide (1) was tested and found to be very slightly inferior to sulphanilamide but far less toxic. That the presence of the guaiacol residue does not influence the therapeutic activity is shown by the fact *p*-[3'-methoxy-4'-hydroxybenzylamino]benzenesulphonamide (6) is found to be far inferior to the parent *p*-benzylaminobenzenesulphonamide (' Proseptasine ').

The results so far obtained indicate that the substitution in the amino part of the molecule in sulphanilamide does not greatly enhance the activity (Buttle, 1939*a*, *b*; Marshall, *loc. cit.*). Of such amino substituted compounds, the Schiff's bases appear to be the most active and least toxic. It is, however, to be settled whether the activity of these compounds is due to the hydrolysis to sulphanilamide *in vivo* and whether their apparent low toxicity is due to the poor absorption in the system as a result of their low solubility (Feinstone *et al.*, 1938).

In compounds with acid groupings as substituents, active derivatives appear to be produced only among derivatives of *p*-aminobenzenesulphonanilide with carboxyl- or sulphonic acid groupings in the second benzene ring in the ortho position. Thus N-sulphanilyltaurine (11) and N-sulphanilylglycine are devoid of activity (Bauer and Rosenthal, 1938; Cooper *et al.*, 1938), while N-sulphanilyl-(β)-phenyl-(α)-alanine (5) is about half as active as sulphanilamide. Crossley *et al.* (*loc. cit.*)

have reported that 2-N¹-sulphanilamidobenzoic acid (4) is superior to sulphanilamide but we find it to be only inferior. It is unexpected that 3-N¹-sulphanilamidocinnamic acid (12) is devoid of activity, while cinnamic acid radical by itself has been claimed to possess strong bactericidal properties (Jacobson, 1929). Crossley *et al.* (*loc. cit.*) have recorded that 4-N¹-sulphanilamidonaphthalene 1-sulphonic acid is as active as sulphanilamide, 2-sulphanilamidonaphthalene 5-sulphonic acid is slightly inferior and 2-sulphanilamidonaphthalene 6-sulphonic acid is toxic. We find that the presence of one more sulphonic acid grouping (13 and 14) and an additional hydroxyl grouping (15 and 16) in such compounds destroys the activity.

There is some difference of opinion regarding the thesis that ' Prontosil ' and related azodyes act only by being broken down in the system to sulphanilamide (Long and Bliss, 1937 ; Rosenthal *et al.*, *loc. cit.* ; Feinstone *et al.*, *loc. cit.* ; Fuller, 1937 ; Domagk, 1936, 1937*a. b* ; Buttle, 1939*a, b*). This assumption requires that the action of the azodyes carrying the benzenesulphonamide group should persist in all the dyes irrespective of the nature of the other component, provided the latter does not hinder the postulated reduction of the azo linkage in the body. Trefouel *et al.* (1937*b*) studied a series of azodyes of the Prontosil group and have found that their activity does change according to the nature of the substituents in the benzene ring of the coupling component. We also find that while the dyes (2 and 3) obtained by diazotizing sulphanilamide and coupling with 4-aminouracil and 4-aminothiouracil respectively are just as active as sulphanilamide, that obtained by coupling with abietic acid (10) is inactive. Similarly, the dyes obtained by diazotizing sulphanilamide and coupling with *p*-hydroxybenzenesulphonamide, 8-hydroxyquinoline (McLeod, 1938), and 2 : 6-diaminopyridine (Fosbinder and Walter, 1939) possess no activity. These indicate that the therapeutic effect of the dyes of this group cannot as a rule be traced to their reduction to sulphanilamide.

The striking therapeutic properties of 2-sulphanilamidopyridine (M. & B. 693) gave the impetus to search for active compounds among the quinoline, isoquinoline, and acridine groups. 6-N¹-sulphanilamidoquinoline (17) was extremely toxic (about 40 times as much as sulphanilamide) and in 0·5 mg. doses it was inactive. De and Basu (1938) have also reported that 8-N¹-sulphanilamidoquinoline is extremely toxic, its therapeutic properties being negligible. These derivatives with substituents in these two active positions of quinoline having proved to be of no use, it appears doubtful whether other quinoline or even isoquinoline derivatives may be of any use at all.

SUMMARY.

1. The implications of the synthesis and study of derivatives related to sulphanilamide have been outlined.

2. The results of testing seventeen compounds related to sulphanilamide in *streptococcal* (hæmolytic) and *pneumococcal* (type I) infections in mice are given. In *streptococcal* infections, three compounds have been found to be slightly less

active than sulphanilamide but far less toxic. 2-sulphanilamidothiazol showed striking activity against the *streptococci* and *pneumococci* comparable to that of 2-sulphanilamidopyridine (M. & B. 693).

3. The relation of chemical constitution to therapeutic action as revealed by a study of the above compounds has been pointed out.

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TOXÆMIAS OF PREGNANCY.

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THIS paper is based on biochemical, dietetic, and clinical studies of 75 cases showing albuminuria during the last trimester of pregnancy. It also includes parallel observations made on a control series of 25 normal gravidæ at about the same period of gestation. The 75 cases studied fell into the following seven groups :—

1. *Eclampsia.*—These showed signs and symptoms of severe and acute toxæmia. Fifteen out of the 17 cases belonging to this group had a sudden onset and severe convulsions.

2. *Nephritic toxæmia.*—These showed signs and symptoms of chronic renal failure (13 cases).

3. *Albuminuria with toxic injury to liver.*—These showed signs of severe toxæmia, albuminuria with jaundice (8 cases).

4. *Albuminuria with pyelitis complicating pregnancy.*—(2 cases).

5. *Albuminuria with diabetes mellitus.*—(2 cases).

6. *Albuminuria with malignant malaria.*—(1 case).

7. *Albuminuria of unknown origin, with or without mild symptoms of toxæmia.*—(32 cases).

Biochemical examination of blood included, estimation of total protein, urea nitrogen, non-protein nitrogen, creatinine, uric acid, sugar, calcium, inorganic

phosphorus, hæmoglobin, van den Bergh reaction, and pH. In addition, in several cases urea clearance was carried out. For purposes of the above estimations blood was withdrawn from the patient's arm and it was citrated. Urea was determined by the urease titration method. Cholesterol by May and Wardell's method using whole blood and pH determination was colorimetric. Calcium was determined by the Clark and Collip modification of Kramer and Tisdall's method ; phosphorus was determined by Bodansky's method. For the estimation of remaining constituents, a protein-free filtrate was obtained from the whole blood by the method of Folin and Wu and determination made by the Folin-Wu system. Wherever possible a second sample of venous blood was obtained after delivery. In a few cases a third sample was also taken when the patient was well on the road to recovery. In cases who were acutely ill and died a few hours after admission, a second determination was not possible. Excretion of vitamin C in the urine was made, using 2 : 6 dichlorphenolindophenol-titration method. A full chemical and microscopic examination of the urine was done in each case. A blood smear was also examined from each case.

The dietary habits of the patients were inquired into soon after their admission to hospital, either from the patients or their attendants. The following points were particularly noted in each case :—

> (*i*) Quantity of food consumed.
> (*ii*) Number of meals per day.
> (*iii*) Addiction to tobacco, betel, alcohol, or opium.
> (*iv*) Quantity and type of fat consumed.
> (*v*) Quantity of carbohydrates consumed.
> (*vi*) Quantity and type of proteins consumed—animal and vegetable.
> (*vii*) Quantity of fruits taken.
> (*viii*) The social position of the patient.

In each case a thorough physical examination was made which not only provided clinical data for the inquiry but also helped in assessing the nutritional state of the patient. It must be stated here that remarks offered on the diet of cases studied are not based on weighment of food consumed but on the approximate weights of different constituents of food as stated by the patients or their attendants. Practically all the patients, toxæmic as well as the controls studied, belonged to the social stratum which seeks admission to the non-paying wards of State Hospitals or attends at Health Centres. The diet of women of this class in the Punjab whether they be vegetarians or non-vegetarians consists mostly of carbohydrate-rich cereals, particularly atta (wheat flour), of which an adult will consume 12 to 14 ounces every day. Rice is taken but rarely and usually at the expense of the atta ration. About two ounces of pulses (dhall) are also taken. Even in non-vegetarian Hindu homes women rarely take meat and amongst the poor Mohammedans, the quantity of meat purchased for the family as a whole is so small that the wife's share would hardly be an ounce or so and that even not daily. On an average she would get $\frac{1}{4}$ to $\frac{1}{2}$ ounce of ghee and a similar quantity of vegetable

oils. About four ounces of vegetables will be consumed and the proportion between green leafy vegetables and other ordinary vegetables, like potatoes and onions, varies. The consumption of curds would hardly come to one ounce per day. For vegetarians average amount of milk or butter-milk consumed would come to about 4 ounces per day and of raw vegetables and fresh fruit together about two ounces per day and that too not all the year round. The diet of cases from rural areas will contain practically no meat or fruit but will be richer in butter-milk. Taking all the year round a greater variety and quantity of vegetables are consumed in the urban than in the rural areas. The consumption of gurh or sugar would be from ½ to 1 ounce per day. The diet of both the controls and the toxæmic cases was poor in 'protective foods'. Milk and milk products, fruit and to a lesser extent green vegetables, were consumed in insufficient quantities and eggs were taken but very rarely.

The foregoing diet represents on the whole about 2,000 to 2,200 calories. It indicates the food consumed by the class of women who constituted the controls in this investigation, a class which represents economically the great bulk of the female population of Northern India. The diet of the majority of the toxæmic cases studied fell short of this standard in caloric value and in the amount of protective foods consumed.

Tables I to IV give the main data gathered in this investigation (*see* end of paper).

Important findings in the groups above mentioned and in the normal controls were as follows :—

Group I. Eclampsia.—In this group of seventeen cases there was no nitrogen retention in the blood, although figures for urea nitrogen, non-protein nitrogen, creatinine, and uric acid, were higher than in the normal controls, yet they were within the normal range. Total proteins were considerably depressed. Serum calcium showed a marked fall. The urine in all cases showed a good concentration as evidenced by normal specific-gravity figures and urine urea. Ten cases showed no vitamin-C excretion in the urine, and in seven the excretion was markedly sub-normal.

Ten patients were vegetarians and seven were mixed eaters. The diet ordinarily taken before illness was ill balanced in every case, carbohydrates preponderating over other constituents. The quantity of food ingested for several months preceding illness was short even of caloric needs approximately 1,300 calories per day. The proteins consumed were of a low biologic value and quantitatively inadequate. Fresh fruit was absent from the dietary of all these cases. The average intake of proteins in the case of vegetarians was 38 g. per day and in the case of mixed eaters 50 g. Nine patients could not afford milk and had not taken it for months. One of these showed carpo-pedal spasm and other signs of tetany. Serum calcium in this case stood at 7·9 mg. per cent. None of the patients were drug addicts. All patients had spongy gums and some carious teeth.

Maternal mortality in this group was 16·6 per cent and infant mortality 50 per cent.

Group II. Nephritic toxæmia.—There were thirteen cases in this group. The patients were a little older on the average than the eclamptic cases. Biochemical findings showed nitrogen retention in the blood ; urea N., N. P. N., creatinine, and uric acid, reached their highest limits in this group. Total proteins showed diminution but stood at a higher level than that found in the eclamptics ; serum calcium was below normal but stood at a higher level than what was found in the eclamptics. Urea-clearance test was done on nine cases and showed uniformly low clearance values. Patients were more anæmic than the eclamptics.

The specific gravity of the urine in these cases was low. Urine urea was much below normal. Albumin was found in smaller quantities (0·184 per cent) in the urines of this group than in the eclamptic. Six cases showed no excretion of vitamin C in their urine and seven had a sub-normal excretion.

Diet consumed by this group was a little better than that consumed by the eclamptic group in that approximately four ounces of milk or curds per day were consumed by almost every case in this group. Average protein in the diet was 50 g. per day and the average caloric value of the diet 1,660. Previous history of renal damage was elicited in all but two cases. Maternal mortality was 18·8 per cent, infant mortality 45·45 per cent.

Group III. Albuminuria with toxic injury to liver.—In this group of eight cases there was no nitrogen retention in the blood. Urea nitrogen, N. P. N., creatinine, and uric acid, were all within the normal range being higher than in the normal controls but lower than in the eclamptic and nephritic groups. Total proteins were present in about the same concentration as in the control group. Blood calcium, although lower than in the normal controls, was decidedly higher than in the nephritic and eclamptic groups. Blood pressure too was lower in this group than in the preceding two groups. Hæmoglobin values were higher in this group than in the preceding two groups. There was more acidosis in this group and the pH stood at 7·4.

Urine urea and the specific gravity of urine were within the normal limits. Two cases showed excretion of leucine and tyrosine crystals in their urine. They died of acute yellow atrophy of the liver as revealed by post-mortem examination. In no case of this group was vitamin C present in the urine.

Diet consumed by patients in this group prior to development of toxæmia was practically of the same caloric value and protein content as that consumed by the controls.

Maternal and infant mortality was 60 per cent.

Group IV. Albuminuria with pyelitis.—There were two cases in this group. There was no nitrogen retention in the blood. Total proteins stood at the same level as in the normal controls. There was hypo-calcæmia and a slight rise in blood phosphorus. The specific gravity of urine was normal. There was no excretion of vitamin C in the urine. The diet was poor in calories (average 1,260) and particularly in ' protective foods '.

Group V. Albuminuria with diabetes mellitus.—There were only two cases. Both were drowsy and on admission showed albuminuria and died in coma. The

diagnosis of diabetes was made on biochemical findings. One of these cases had had three pregnancies—first child still-born, second delivery by craniotomy, third by Cæsarean section. Biochemical investigation showed no nitrogen retention in the blood. Blood proteins were present in nearly the same concentration as in the control group. Blood sugar and blood cholesterol were high in both cases. Serum calcium was low in one case but was normal in the other. Phosphorus was very much the same in both. The cases were definitely anæmic. Acidosis was present, the pH standing at 7·4. There was acetonæmia—the total acetone bodies in the blood standing at 5 mg. and 6 mg. per cent. Blood films did not show any hæmoparasites. Specific gravity of urine was much raised. Urine contained small quantity of albumin. Urine urea showed a normal concentration in one and a low concentration in the other. Urine sugar was 1·5 per cent and 1·8 per cent, respectively. There was marked acetoneuria, indicanuria, and urobilinuria. There was no excretion of bilirubin or bile salts in the urine. The dietetic history of both cases pointed to a diet rich in carbohydrates but poor in protein and mineral content.

Group VI. Albuminuria with malignant malaria.—There was only one case. The patient was admitted as a case of pregnancy toxæmia. She had albuminuria, generalized anasarca, marked anæmia, and spongy pyorrhœaic gums. Her blood pressure was 110/75. She had a systolic hæmic murmur and enlarged liver and spleen. She became drowsy, comatose, and died. Her blood film showed many malignant tertian rings. There was no vitamin-C excretion in the urine. Indican uria and urobilinuria were present.

She was a poor woman and subsisted mainly on atta, salt, and pepper.

Group VII. Albuminuria of unknown origin.—In this group of thirty-two cases, there was no hypertension and no nitrogen retention in the blood. Serum calcium and phosphorus were about normal. Total proteins were below normal. Twenty-five patients suffered from nutritional anæmia of pregnancy. The hæmoglobin in these cases was either 50 per cent or below, the lowest figure being 35 per cent. Urea-clearance test was performed in two cases and a mild renal deficit was found in each. Vitamin-C excretion was normal in three cases, sub-normal in seventeen cases, and no excretion in twelve cases.

The caloric value of the diet consumed averaged 1,600 per day and was relatively poor in 'protective foods'.

Maternal mortality in this group was 5·88 per cent. Infant mortality was 10·5 per cent.

The diet of the controls (women attending Health Centre) was also poor in 'protective foods' and not properly balanced particularly in respect of animal proteins. Figures for total protein and hæmoglobin were lower than those quoted in the West, and average hæmoglobin content of blood in this group was 10·6 mg. per cent.

DISCUSSION.

Toxæmias of pregnancy are of fairly common incidence in India. Of 663 labour cases treated in the Lady Willingdon Hospital, Lahore, during 1937, as

many as 108, viz., 16·4 per cent showed signs of toxæmias. Of this number 14 or 2·1 per cent were cases of eclampsia. The incidence of eclampsia in Bombay according to Mehta (1936) is 0·05 per cent, in Madras it is 1·75 per cent as recorded by Hingston and Mudaliar (1927), and according to Balfour (1927) it is 4·1 per cent in Calcutta. The Punjab appears to occupy a position mid-way between Madras and Calcutta in the matter of incidence of eclampsia and the same may be true of the incidence of toxæmias of pregnancy.

In spite of an enormous amount of investigation, the ætiology of this important morbid condition is still obscure. The investigation under report financed by the Indian Research Fund Association was undertaken with a view to find out whether a study of the diet and the chemistry of blood and urine of pregnant women in India could throw any light on the ætiology of these disorders. The biochemical findings of the 25 controls given in Table II show that our figures for the total proteins of blood and for calcium are lower than those in the West and the same is true of hæmoglobin. The relative deficiency noticed in the controls is aggravated in cases of toxæmias. Ill-balanced and inadequate diets may arise as much out of want, as from ignorance and bad habits. The deficiencies observed in the poorer class of cases studied may also be found amongst the well-to-do people due to lack of care in diet construction. That this investigation was apparently concerned with the class of patients treated free of charge in State Hospitals must not suggest that the toxæmias of pregnancy only affect the poor.

Are these deficiencies causally related with manifestations of toxæmias of pregnancy ? The information obtained regarding the diet of cases studied showed such imbalance and deficiencies as could account at least for some of these manifestations. The diet was deficient in protein, calcium intake, and vitamins, even in controls and more so in the toxæmic group. Hypo-proteinæmia can account for œdema and possibly some of the symptoms of encephalopathy as has been shown by Strauss (1938). McIlory (1936) quotes Stroganoff who emphasizes the importance of myoneural irritability and vaso-spasm ; mentioning hypo-calcæmia as one of the predisposing factors. Sub-normal serum calcium values in eclampsia and to a lesser extent in nephritic and pre-eclamptic toxæmia in England were reported by Anderson (1932) and this investigation has given similar results in the case of Indian women. Low hæmoglobin by causing injury to the renal filter, through anoxæmia, may account for albuminuria, a fact also stressed by McCay (1912) while discussing the effect of low protein dietary in Bengal. Renal ischæmia resulting from vaso-spasm may also cause the appearance of albumin in urine. McIlory (*loc. cit.*) quotes de Wesselow and Wayatt as saying that generalized vaso-constriction affecting the kidneys causes local ischæmia and parenchymatous changes.

These and other defects of the biochemical picture could arise from quantitative and qualitative malnutrition or both. Particularly important in this connection would be sequelæ of hypo-vitaminoses B_1 and C.

The emphasis laid in some quarters on the importance of the internal secretions in the ætiology of toxæmias of pregnancy could reasonably be shifted to the plane of dietetics. The rôle of different vitamins in promoting the function of

different endocrine glands has been brought out by recent investigation. The probable influence of vitamin B_1 on the optimal functioning of the pituitary as mentioned by Siddall (1938); the relationship between vitamin B_1 and thyroid activity as reviewed by Carpenter and Sharpless (1937); the presence of vitamin C in high concentration in the adrenal, the pituitary and the corpus luteum, the probable mode of action of vitamin D through parathyroid stimulation as shown by Taylor et al. (1932); the possible relationship of vitamin E to anterior hypophysis and thyroid as shown by Rowland and Singer (1936) and Singer (1936), and finally the relationship of vitamin E to gonadal activity, all point to the importance of the dietary factor in the ultimate causation and control of disorders like the toxæmias of pregnancy, be it through the endocrine mechanism or through factors outside the range of hormonic influence. The association of hypo-vitaminosis B_1 with the ætiology of toxæmias of pregnancy and the effect of vitamin D and calcium therapy in pregnancy toxæmias has been studied by Siddall (*loc. cit.*) and Theobald (1937), respectively. In another paper Theobald (1936) associates the development of toxæmic symptoms with a meat diet, a disturbance of calcium metabolism, and an insufficient intake of vitamin-B complex. The almost consistent finding in the cases studied of complete or almost complete disappearance of vitamin C in the urine, is a finding of great importance. Vitamin C is one of the most active catalytic and de-toxicating agents in the body. The human subject is incapable of synthesizing it in the body and it must be supplied through ingested food. Its deficiency in all probability plays an important rôle in the causation of toxæmias of pregnancy. Metabolic disorders of known and unknown ætiology injure important organs like the liver and kidneys. Infections and sub-infections complicate the picture of toxæmias and create the many subdivisions of this disorder recognized in the textbooks, but behind all these complications must be recognized the common factor of defective or disordered nutrition which in all probability sets the stage for toxæmias of pregnancy. The appreciation of this factor could help greatly in preventive efforts against these disorders.

SUMMARY.

1. A full clinical examination and a detailed biochemical examination of blood and urine was carried out in each of the seventy-five cases suffering from toxæmias of pregnancy.

2. Parallel observations were made on twenty-five normal pregnant controls at about the same period of gestation.

3. Dietary habits of the cases studied were also noted.

4. Hypo-proteinæmia and hypo-calcæmia were the main positive findings with regard to the blood in the cases studied particularly in those belonging to the eclamptic and nephritic groups. The urine showed poor excretion or absence of vitamin C.

5. The diet was deficient in 'protective foods', especially from the point of view of minerals, like calcium, and of the vitamins.

6. The importance of disordered or defective nutrition in the probable causation of pregnancy toxæmias is stressed.

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TABLE I.

Symptomatology.

Number of cases.	Type.	Religion. H.	M.	S.	C.	Age.	Primi or Multigravida. P.	M.	Blood pressure. Max.	Min.	Mean.	Albuminuria. Present in number of cases.	Œdema. Present in number of cases.	Headache. Present in number of cases.
17	Eclampsia ..	7	6	3	1	16—32	13	4	$\frac{220}{120}$	$\frac{105}{70}$	$\frac{150}{105}$	17	17	11
13	Nephritic toxæmia ..	6	5	..	2	18—37	5	8	$\frac{242}{130}$	$\frac{95}{60}$	$\frac{145}{95}$	13	13	12
8	Albuminuria with toxic injury to liver.	3	..	5	..	16—30	5	3	$\frac{120}{70}$	$\frac{74}{40}$	$\frac{96}{65}$	8	1	5
2	Albuminuria with pyelitis.	1	1	..	..	21—22	..	2	$\frac{120}{80}$	$\frac{118}{70}$	..	2	2	..
2	(a) Albuminuria with diabetes.	2	..	..	..	19—22	1	1	$\frac{123}{95}$	$\frac{90}{60}$	..	2	2	..
1	(b) Albuminuria with malignant malaria.	1	..	..	..	..	..	1	$\frac{110}{70}$	..	..	1	1	..
32	Albuminuria of unknown origin.	21	8	1	2	19—30	12	20	$\frac{140}{96}$	$\frac{90}{60}$	$\frac{105}{75}$	32	32	5

H.—Hindu. M.—Mohammedan. S.—Sikh. C.—Christian.

Table I—*concld.*

Number of cases.	Type.	Vomiting. Present in number of cases.	Giddiness. Present in number of cases.	Visual disturbances. Present in number of cases.	Convulsions. Present in number of cases.	Drowsiness. Present in number of cases.	Coma. Present in number of cases.	Jaundice. Present in number of cases.	Mental symptoms. Present in number of cases.
17	Eclampsia ..	11	5	4	12	15	8	..	6
13	Nephritic toxemia ..	5	7	6	7	6	8	..	3
8	Albuminuria with toxic injury to liver.	1	..	..	..	7	6	8	..
2	Albuminuria with pyelitis.	..	..	..	..	..	..	..	..
2	(a) Albuminuria with diabetes.	..	..	..	..	2	2	..	..
1	(b) Albuminuria with malignant malaria.	..	..	..	..	..	..	..	..
32	Albuminuria of unknown origin.	4	..	..	..	..	..	..	..

TABLE II.

Chemical analysis of blood in 75 cases of pregnancy toxæmia and 25 pregnant controls.

Number of cases.	Type.	UREA MG. PER CENT.			N. P. N. MG. PER CENT.			UREA N./N. P. N.		
		Max.	Min.	Mean.	Max.	Min.	Mean.	Max.	Min.	Mean.
17	Eclampsia	25	12·5	18·55	46	28	34	0·66	0·41	0·54
13	Nephritic toxaemia	47·5	18·8	28·78	63	23	43·3	0·75	0·58	0·663
8	Albuminuria with toxic injury to liver.	25	13	17·3	44	20	30	0·71	0·45	0·58
2	Albuminuria with pyelitis	25	24	24·5	44	40	42	0·65	0·55	0·587
2	Albuminuria with diabetes	27	12	19·5	50	26	38	0·54	0·46	0·5
1	Albuminuria with malignant malaria.	..	..	10	..	..	24	..	..	0·316
32	Albuminuria of unknown origin.	28	11	17·05	41	22	29·85	0·69	0·33	0·567
25	Normal controls	18	12	14·5	40	24	30	0·48	0·43	0·47

Note.—In two cases of albuminuria with diabetes total acetone bodies in the blood stood at 5 mg. and 6 mg. per cent respectively.

TABLE II—*contd.*

Number of cases.	Type.	Creatinine mg. per cent.			Uric acid mg. per cent.			Total protein per cent.		
		Max.	Min.	Mean.	Max.	Min.	Mean.	Max.	Min.	Mean.
17	Eclampsia	3	1	2·1	7	2	4·21	6·1	3·4	4·3
13	Nephritic toxæmia ..	..	1·13	2·67	6	1·13	2·67	5·9	4·5	5
8 {	Albuminuria with toxic injury to liver.	3	1·5	2	5·5	2·5	2·75	5·82	5·65	5·75
2	Albuminuria with pyelitis	2·2	1·8	2·0	4·35	3	3·775	5·76	5·72	5·74
2	Albuminuria with diabetes	2·4	2	2·2	3	3	3	5·9	5·5	5·7
1 {	Albuminuria with malignant malaria.	..	..	1·3	..	..	2·3	..	..	5
32 {	Albuminuria of unknown origin.	2·3	1·3	1·75	5·5	3	4·11	6·3	4·3	5·47
25	Normal controls ..	1·8	1·2	1·5	3·1	1·7	2	6·2	5·74	5·8

Note.—In two cases of albuminuria with diabetes total acetone bodies in the blood stood at 5 mg. and 6 mg. per cent respectively.

TABLE II—*contd.*

Number of cases.	Type.	SUGAR MG. PER CENT.			CHOLESTEROL MG. PER CENT.			CALCIUM MG. PER CENT.		
		Max.	Min.	Mean.	Max.	Min.	Mean.	Max.	Min.	Mean.
17	Eclampsia ..	190	80	129·9	230	151	185·1	9	7·6	8·27
13	Nephritic toxaemia ..	200	75	115·6	227	150	185·7	9·45	6	8·36
8 {	Albuminuria with toxic injury to liver.	119	103	111·4	250	192	222·4	9·2	8·2	8·71
2	Albuminuria with pyelitis	125	89	112	180	169	174·5	8·6	8·25	8·425
2	Albuminuria with diabetes	210	190	200	268	210	239	9·30	8·25	8·7
1 {	Albuminuria with malignant malaria.	..	..	110	..	..	176	..	..	..
32 {	Albuminuria of unknown origin.	183	97	122·93	225	110	183·05	9·3	8	8·89
25	Normal controls ..	135	109	111	201	160	175	9·7	8·9	9·2

Note.—In two cases of albuminuria with diabetes total acetone bodies in the blood stood at 5 mg. and 6 mg. per cent respectively.

TABLE II—*contd.*

Number of cases.	Type.	Phosphorus mg. per cent.			Hæmoglobin per cent.			pH.		
		Max.	Min.	Mean.	Max.	Min.	Mean.	Max.	Min.	Mean.
17	Eclampsia ..	.	3	4·1	80	36	66·5	7·52	7·4	7·45
13	Nephritic toxæmia ..	6	3	4·05	80	50	64·8	7·5	7·39	7·44
8	Albuminuria with toxic injury to liver.	3·5	3·5	3·5	80	68	72	7·46	7·27	7·4
2	Albuminuria with pyelitis	4·26	3·9	4·01	80	67	73·5	7·49	7·47	7·48
2	Albuminuria with diabetes	4·25	4·1	4·175	70	60	65	7·4	..	..
1	Albuminuria with malignant malaria.	..	..	4·1	..	..	50	7·5	..	..
32	Albuminuria of unknown origin.	5	3·7	4·09	85	35	63·68	7·53	7·41	7·48
25	Normal controls ..	3·8	3	3·5	90	76	80	7·52	7·49	7·5

Note.—In two cases of albuminuria with diabetes total acetone bodies in the blood stood at 5 mg. and 6 mg. per cent respectively.

TABLE II—*concld.*

Number of cases.	Type.	Urea clearance.			van den Bergh reaction.
		Max.	Min.	Mean.	
17	Eclampsia	52·15	U/B	30	Indirect + in 3 cases.
13	Nephritic toxæmia ..	53·2	16·9	33·37	Indirect + in 3 cases; direct in one case.
8	Albuminuria with toxic injury to liver	..	..	..	Indirect + 1; direct + 1; biphasic — 3.
2	Albuminuria with pyelitis ..	..	..	..	Negative.
2	Albuminuria with diabetes ..	..	..	..	Indirect + in one; delayed direct + in one.
1	Albuminuria with malignant malaria ..	..	..	..	Indirect +.
32	Albuminuria of unknown origin ..	65	50	On 2 cases only.	} Indirect + in 7 cases.
25	Normal controls ..	Done only in one case			Negative.

Note.—In two cases of albuminuria with diabetes total acetone bodies in the blood stood at 5 mg. and 6 mg. per cent respectively.

TABLE III.

Statistical analysis.

Type.	Total proteins of blood per cent.	Total calcium in blood mg. per cent.
Eclampsia	4·3 ± 0·8	8·27 ± 0·3
Nephritic toxæmia ..	5 ± 0·4	8·36 ± 0·3
Albuminuria with toxic injury to liver.	5·75 ± 0·07	8·71 ± 0·4
Albuminuria with pyelitis ..	5·74 ± 0·02	8·425 ± 0·175
Albuminuria of unknown origin	5·47 ± 1·1	8·89 ± 0·8
Normal controls	5·8 ± 0·04	9·2 ± 0·2

Table IV.

Chemical examination of urine. Seventy-five cases of pregnancy and 25 normal pregnant controls.

Number of cases.	Type.	Specific gravity.			Urea percentage.			Albumin percentage.			Indican.		Urobilin.		Sugar percentage.
		Max.	Min.	Mean.	Max.	Min.	Mean.	Max.	Min.	Mean.	Present in number of cases.	Absent in number of cases.	Present in number of cases.	Absent in number of cases.	
17	Eclampsia ..	1040	1010	1022	4	1·2	1·8	1·91	Trace	0·4	9	8	10	7	..
13	Nephritic toxæmia ..	1019	1003	1009	2·4	0·3	1·1	1	0·005	0·1	6	7	8	5	..
8	Albuminuria with toxic injury to liver.	1022	1010	1015	2·8	0·7	1·9	..	Trace	..	8	..	8	..	..
2	Albuminuria with pyelitis.	1016	1014	1015	1·2	0·3	0·75	0·2	0·05	0·125	..	2	2	..	..
2	Albuminuria with diabetes.	1020	1019	1019·5	2·5	0·8	1·65	0·01	0·0065	0·0075	2	..	2	..	1·8 / 1·5
1	Albuminuria with malignant malaria.	1010	..	..	2·1	..	..	0·05	..	..	..	1	1	..	..
32	Albuminuria of unknown origin.	1028	1010	1018	4	0·5	2	0·15	0·005	0·08	19	13	20	12	..
25	Controls ..	1018	1012	1015	3·21	2·5	2·8	..	..	..	..	15	10	..	..

SOME OBSERVATIONS ON THE INHIBITION OF ADRENALINE OXIDATION.

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ADRENALINE, as judged by the fading out of its pressor action, has long been known to be rapidly destroyed *in vivo*. If the mechanism of this destruction could be defined, we would be enabled to devise means to prolong the biological activity of this hormone in the organism.

A study of the behaviour of the salts of adrenaline in aqueous solution to ærobic oxidation might be counted among the first attempts to understand the mechanism destroying the drug. Cumulative evidence points to the fact that an alkaline pH (Sugawara, 1928-29) and metallic ions, notably Cu and Fe (Barker *et al.*, 1932; Schild, 1933), catalyse the rapid oxidation of adrenaline.

In vitro experiments have revealed that the capacity which most tissues and tissue fluids possess to catalyse the oxidation of adrenaline, differs widely. Thus, while the mammalian vascular tissue greatly accelerated adrenaline destruction *in vitro* (Tatum, 1912), blood serum and plasma have been found to do so in a very much diminished degree due to adsorption and fixation of adrenaline by corpuscles in the whole blood (Bain *et al.*, 1937).

Similarly, it has been recorded by several investigators that, after intra-arterial injection, adrenaline is rendered biologically inert while circulating through the liver or intestines.

Growing literature on the subject tends to the view that the destruction of adrenaline by animal tissues might be traceable ultimately to the specific enzymes therein, especially phenolases and/or amine oxidase. It is known now that both these types of enzymes oxidize adrenaline, although in quite different manners. The phenolases oxidize the hydroxyls in the catechol residue (Neuberg, 1908;

Heard and Raper, 1933), and the amine oxidase the side chain (Richter, 1937) of the adrenaline molecule. To the former category must be added the cytochrome-indophenol system, so common in all animal tissues. The mechanism of this type of oxidation according to Green and Richter (1937) is as follows :—

(ORTHOQUINONE.)   (ADRENOCHROME.)
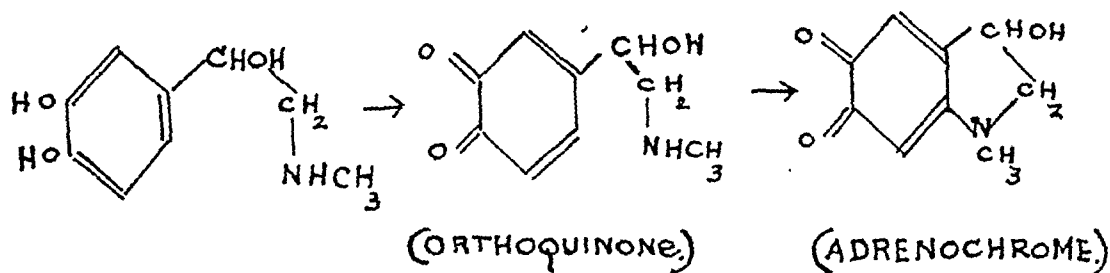
Studies on the occurrence and distribution of phenolases (Bhagvat and Richter, 1938) and amine oxidase (Blaschko *et al.*, 1937) in the animal organism suggest that among the varied physiological rôles of these enzymes, one might be the oxidation of adrenaline. A significant observation has been made by Weinstein and Manning (1937) that a substance with the properties of protocatechuic acid appeared in the urine of rabbits to which adrenaline had been administered. It would appear, therefore, that the adrenaline molecule is oxidized in the side chain by an enzyme, probably amine oxidase.

With the development of our knowledge that the destruction of adrenaline *in vivo* is essentially an enzymatic process, protector mechanisms capable of inhibiting this process have come to light. In his experiments on hexuronic acid (ascorbic acid) then isolated by him, Szent-Györgyi (1928) showed that the pigment formed by the oxidation of adrenaline by a peroxidase system was prevented effectively by the presence of ascorbic acid. When this vitamin was all oxidized in the system, only then did the normal oxidation of the hormone start. Similarly, the capacity of adrenaline to act as a carrier in the coupled oxidation of substrates with catechol oxidase has been demonstrated by Richter (1934). The oxidation of ascorbic acid in such a coupled system proceeds at the expense of the adreno-quinone (the o-quinone of adrenaline) which is momentarily formed and as rapidly reduced back to adrenaline. Thus, the action of adrenaline is catalytic. Hence, as long as enough ascorbic acid is available in the reduced state to accept oxygen, adrenaline remains intact. This has been confirmed by Clark and Raventos (1939) who found that the addition of one part per million of ascorbic acid to three parts per 100 millions of adrenaline was sufficient to reduce to one-fourth the rate of oxidation of adrenaline in a bath containing strips of frog's auricle suspended in 10 c.c. of a modified Ringer's solution, as well as in the case of a wetted auricle strip. Bacq (1936), however, found that reduced ascorbic acid which stops oxidation of adrenaline *in vitro*, does not sensitize *in vivo*, and thought that this was due to the instability of ascorbic acid therein. But the correct explanation might be, as also suggested by Iyengar and Mukerji (1939), that the oxidation of adrenaline in the animal body is mostly due to amine oxidase, against which ascorbic acid does not afford protection to the amine. Akin to ascorbic acid,

Bacq (*loc. cit.*) found that a number of other reducing agents, like pyrogallol, also increased the duration of adrenaline action.

As already indicated, amine oxidase disrupts the side chain of adrenaline by oxidative de-methylamination to an aldehyde and methylamine (Richter, 1937). The reaction may be represented by the equation :—

$$HO-C_6H_3(OH)-CHOH\cdot CH_2\cdot NHCH_3 + \tfrac{1}{2}O_2 \longrightarrow HO-C_6H_3(OH)-CHOH\cdot CHO + NH_2CH_3$$
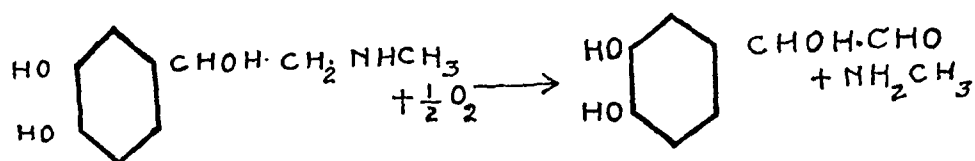
As regards the mechanism of this enzyme action in relation to substrate configuration, Blaschko *et al.* (*loc. cit.*) observed that only compounds containing an amino-group at the end of a hydrocarbon chain were oxidized by amine oxidase, while compounds with a substituent on the α-carbon atom, such as isopropylamine, benzedrine, and ephedrine, were not so attacked. These authors also found that ephedrine, by itself not a substrate for amine oxidase, still had a marked influence in diminishing the activity of the enzyme towards adrenaline *in vitro*.

Gaddum (1938) has attempted to extend the application of the protective action of ephedrine on the system adrenaline-amine oxidase to *in vivo* conditions. In collaboration with Kwiatkowski (Gaddum and Kwiatkowski, 1939), he has obtained experimental evidence which suggests that adrenaline is the chemical transmitter liberated at the neurocytal junction of adrenergic nerves, just as acetylcholine is the chemical transmitter liberated at the neurocytal junction of cholinergic nerves. By an improved method of perfusing the rabbit's ear, these authors showed that stimulation of sympathetic nerves in this preparation caused vasoconstriction, and the liberation of a substance, identified to be adrenaline, by a ·delicate colorimetric test. Ephedrine increased the yield of the substance liberated by the nerve-endings.

Previously, many authors had observed that ephedrine, when given along with or prior to a dose of adrenaline, potentiates or prolongs its action. Thus, Pak and Tang (1933) found that the application of ephedrine to rabbit's eye sensitized the pupil to a subsequent application or injection of adrenaline. On the contrary, ephedrine in high concentrations has been found to antagonize adrenaline action instead of potentiating it (Curtis, 1929).

The short review of literature presented reveals that the process (or processes) by which adrenaline is destroyed in the animal body is very complex and has only been partially understood. It is yet to be shown whether the different enzymes catalysing adrenaline destruction are present and operate individually or collectively at the seat of adrenaline liberation, namely, the adrenergic nerve-endings, or whether they attack the drug in circulation.

Our approach to this problem was initiated by the observation previously made by one of us (J. C. D.) that the rise in blood pressure in cats, when adrenaline was injected after cotarnine, was much higher and more prolonged (Dey *et al.*, 1934). Incidentally, some more experiments in this direction indicated that the

potentiation of adrenaline effect can be produced by cotarnine derivatives as well.

We have now obtained further experimental proofs to show that ephedrine inhibits the enzymatic destruction of adrenaline. This has involved a study of the effects of small doses of ephedrine, such as by themselves would have no appreciable pressor effect, when injected intravenously into anæsthetized and decerebrate dogs, on the destruction of adrenaline in the presence, severally of (I) phenoloxidase as present in a preparation from the seeds of *Dolichos lablab* and (II) amine oxidase from (*a*) defibrinated blood and (*b*) guinea-pig's liver.

Experimental.

I. *Effect of ephedrine on system adrenaline-phenol-oxidase.*

Enzyme.—A phenolase preparation was made according to Bhagvat (1938). Ten grammes of the seed meal of *Dolichos lablab* were extracted with 100 c.c. of a 5 per cent solution of sodium chloride for 24 hours in the ice-chest. The extract was filtered and the clear filtrate used as the source of the enzyme.

Reaction mixtures.—(*a*) To $9\frac{1}{2}$ c.c. of the enzyme preparation was added 0·5 c.c. of 1 in 1,000 adrenaline hydrochloride, so that if 1 c.c. of the finally diluted solution be given intravenously, the dose would be 0·05 mg. of adrenaline hydrochloride. This solution was the control. (*b*) To the same solution in another test-tube was added 2 mg. ephedrine hydrochloride, so that 1 c.c. contained, in addition to 0·05 mg. adrenaline hydrochloride, 0·2 mg. ephedrine hydrochloride. (This dose of ephedrine does not produce any appreciable rise in blood pressure when given intravenously to dogs under anæsthesia). The pH in both the test-tubes was about the same and ranged from 5·9 to 6·1.

The two test-tubes were kept immersed in a thermostat at 38°C. Within a minute a red colour was produced in both the test-tubes and subsequent injection of these solutions into an anæsthetized or decerebrate dog showed a progressive decrease in blood pressure.

Inhibition of oxidase.—If a stabilizer like HCN was used in sufficient concentration, e.g., 10^{-2}M cyanide in the above experiments, the production of red colour was totally inhibited and the original pressor effect of adrenaline was kept up. Similarly, if enough ascorbic acid was added, i.e., 0·35 mg. per c.c., the formation of adrenochrome was again prevented even after 135 minutes. When the HCN concentration was reduced from 10^{-2}M to 10^{-3}M, the colour was produced, but at a slower rate and even slower in the presence of ephedrine.

The observed rapid oxidation of adrenaline in the above experiments and the effective inhibition of such oxidation by ascorbic acid or HCN—either of which does not inactivate amine oxidase—clearly prove that the enzymes herein responsible for the oxidation are the phenolases. Among these, catechol oxidase, present in almost all plant tissues and to a limited extent in animal tissues, is known to oxidize adrenaline directly (Heard and Raper, *loc. cit.*; Bhagvat and Richter, *loc. cit.*) as does tyrosinase (Abderhalden and Guggenheim, 1908). The same effect can

be brought about by indophenol oxidase only through the intermediation of cytochrome (Green and Richter, *loc. cit.*). *Dolichos lablab* is known to contain besides catechol oxidase, tyrosinase (Narayanamurthi and Ramaswami Ayyar, 1929). Hence, the oxidation of adrenaline by the *Dolichos* preparation is to be attributed to catechol oxidase and/or tyrosinase. We cannot, therefore, assume with Iyengar and Mukerji (*loc. cit.*) that the observed effect is due to indophenol oxidase. This enzyme is essentially of animal origin and its presence and that of its essential counterpart, cytochrome, have yet to be shown in *Dolichos lablab*.

Our finding that, in the presence of ephedrine, destruction of adrenaline is slowed in the enzyme mixture has been constant (Graph 1). It is difficult to explain

GRAPH 1.
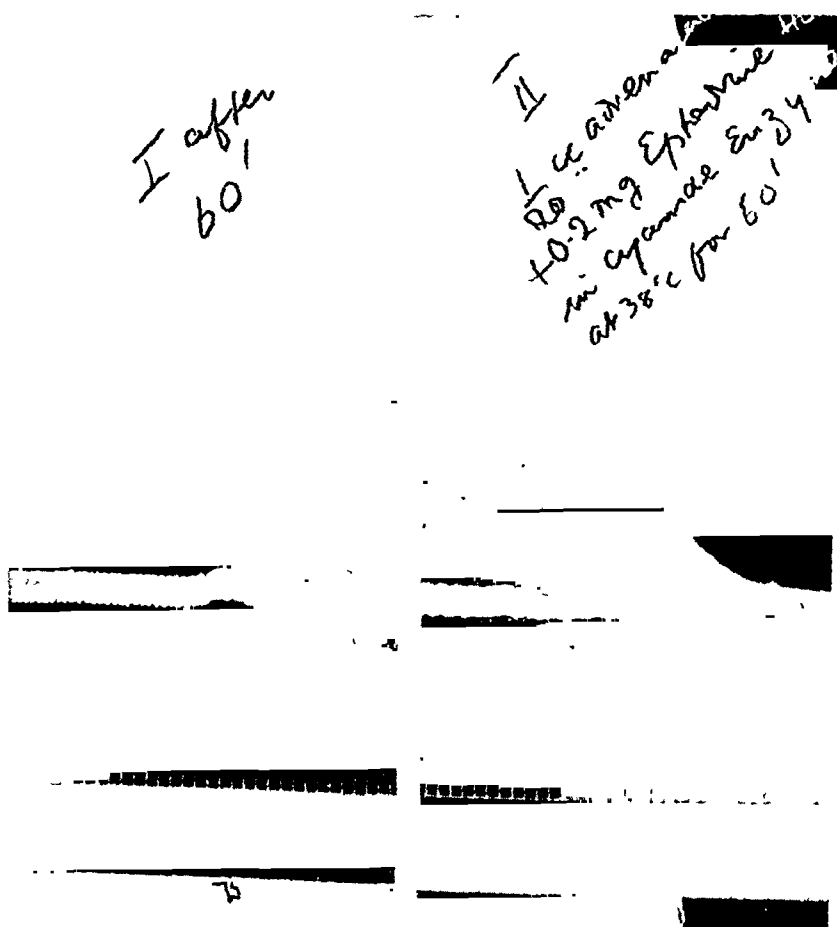
Dog 5·3 kg.—Decerebrate (28–7–1939).
I. Injection of adrenaline (control). Blood pressure after 60 minutes.
II. Injection of adrenaline + ephedrine in cyanide-*Dolichos* enzyme. Blood pressure after 60 minutes.

this observation, unless it be that ephedrine to some extent reduces the enzyme activity of the phenolase also. But, in the absence of cyanide, ephedrine is unable to restrain to any extent the destruction of adrenaline by *Dolichos* enzyme. It may be that, when enough cyanide is present to inactivate most of the phenol oxidase, ephedrine may exert some retarding action on the enzyme. This effect appears to be somewhat similar to the one produced by a mixture of inhibitors first observed by Heard and Welch (1935) and later confirmed by Blaschko *et al.* (*loc. cit.*).

II.   *Effect of ephedrine on the system adrenaline-amine-oxidase.*

(*a*) *Defibrinated blood as source of the enzyme.*—To 18 c.c. of Locke's solution containing defibrinated blood in a test-tube were added 1 c.c. of 1 in 1,000 adrenaline hydrochloride solution and 1 c.c. of M/50 HCN to make up the volume to 20 c.c. so that 1 c.c. of this mixture contained 0·05 mg. adrenaline hydrochloride and 10^{-3}M HCN. The pH of this solution ranged from 7·5 to 7·7. This was the control. To another test-tube containing this solution was added ephedrine hydrochloride to give a concentration of 0·2 mg. per c.c. Both the tubes were kept at 38°C. in a thermostat. Oxygen from a cylinder was passed in a continuous stream for 15 minutes even before the addition of any of the drugs, as it was found that in the absence of oxygenation the destruction of adrenaline was extremely slow (Sugawara, *loc. cit.*). Anæsthetized and decerebrate dogs were given intravenous injections of 1 c.c. each of the control and the ephedrine-containing solutions at intervals of time, and the blood pressure recorded. The ephedrine-containing solution was given only after an appreciable destruction of adrenaline had taken place, for, if ephedrine was given earlier, it often modified the rise in blood pressure due to a subsequent injection of adrenaline.

Graph 2 shows the rise in blood pressure in both cases after incubation for 165 minutes. The destruction of adrenaline is shown to be definitely retarded, but not completely inhibited, by the addition of a small dose of ephedrine which does not by itself have a pressor action. In other cases where the destruction of adrenaline in the control was very slow, ephedrine completely inhibits the destruction of adrenaline. In the experiment illustrated by the graph, the explanation for the incomplete inhibition of adrenaline destruction may be, either HCN was insufficient to inactivate completely the other oxidases present—higher concentrations of HCN tending to kill all enzyme activity, being a protoplasmic poison—or that ephedrine was not in sufficient concentration to inactivate all the amine oxidase present. Another experiment was also done in which M/15 Sørensen's phosphate buffer solution with defibrinated blood was used instead of Locke's solution, putting in instead of HCN, 1 mg. ascorbic acid per c.c. to inhibit the action of other oxidases. The pH of this solution was 7·1 (with Locke's solution and ascorbic acid the pH was too much on the acid side, i.e., about 4·52). The results were identical with those obtained with Locke-blood-HCN mixture.

(*b*) *Guinea-pig's liver as the source of the enzyme.*—A preparation of amine oxidase was made according to Blaschko *et al.* (*loc. cit.*) from guinea-pig's liver. Fifteen grammes of the liver were ground in a mortar with purified sand for

20 minutes and 50 c.c. of M/15 phosphate buffer added and centrifuged for 15 minutes at 1,500 revolutions per minute. To 15 c.c. of the centrifuged liver extract were added 15 mg. of ascorbic acid. To 4·5 c.c. of this solution were

GRAPH 2.
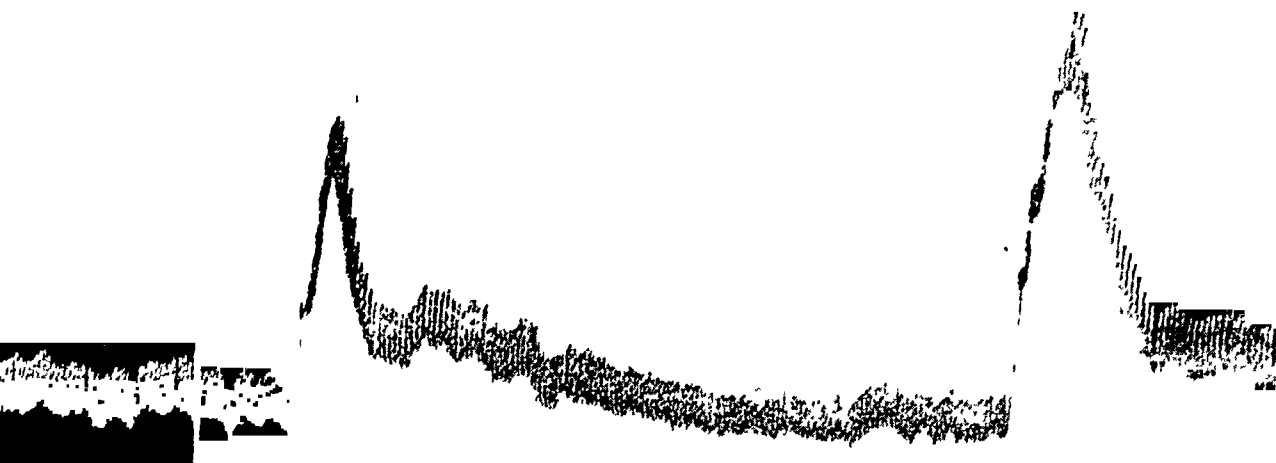
Dog 6·5 kg.—Chloretone and morphia (29–7–1939).
I.   Adrenaline (0·05 mg.) in Locke's with defibrinated blood after 165 minutes (control).
II. Adrenaline (0·05 mg.) + ephedrine (0·2 mg.) in Locke's with defibrinated blood after 165 minutes.
The height of blood pressure with adrenaline (0·05 mg.) alone (not shown) was higher than in II above.

added 0·25 c.c. of adrenaline hydrochloride solution 1 in 1,000 and 0·25 c.c. of distilled water so as to give in each half c.c. 0·025 mg. adrenaline hydrochloride and 0·45 mg. ascorbic acid. This served as the control. Another test-tube contained, in addition to the above mixture, 0·2 mg. of ephedrine hydrochloride per half c.c. of solution. The two test-tubes were immersed in a thermostat at 38°C. Anæsthetized or decerebrate dogs were given intravenous injections, half c.c. each, of the control and the ephedrine-containing solutions at intervals of time and blood pressure was recorded.

Graph 3 represents the results of a typical experiment. From the graph it will be seen that after 120 minutes, more than half of the original quantity of adrenaline is destroyed in the control, whereas in the presence of ephedrine, there is an appreciable, but not complete, retardation of the destruction of adrenaline. Obviously, the amount of ephedrine is not sufficient here to inhibit completely the destruction of adrenaline by amine oxidase present in the liver preparation. We cannot have, however, a larger concentration of ephedrine, as that would produce an additional pressor effect.

In the above experiments II (*a*) and (*b*), the oxidation of adrenaline, both autocatalytic and that due to phenolases, has been inhibited by HCN or ascorbic acid. Hence, the observed destruction of adrenaline in the control solution must be due to amine oxidase present in the preparations. This destruction, as Richter (1937) has shown, is a case of oxidative demethylamination. On the other hand, Clark and Raventos (*loc. cit.*) are inclined to think that the inactivation of adrenaline by frog's auricle is due, chiefly, to the action of some esterase in the tissue. The inhibition of this inactivation by ascorbic acid is explained by them on the grounds that adrenaline and ascorbic acid competed for the same catalyst. This catalyst, therefore, judged by what we know of the nature and extent of protection afforded by ascorbic acid to adrenaline from oxidation, is a phenolase and not an esterase. Further, according to accepted concepts of esterase action, the adrenaline molecule provides no scope whatsoever to act as a substrate for an esterase. Clark and Raventos seem to have based their views, as Gaddum (*loc. cit.*) has done *in extenso*, on the analogy of the system acetylcholine-esterase. Excepting that adrenaline and acetylcholine are said to be liberated at and act on nerve-endings, there is nothing else in common between them, especially in the manner of their destruction. While the destruction of acetylcholine by esterase is an individual and, therefore, simple process of hydrolysis, that of adrenaline, either by phenolase or amine oxidase, is a process of oxidation, involving a series of complex changes in the adrenaline-phenolase system. In the light of this, it is only to be anticipated, as has been found to be the case by Clark and Raventos, that the cumulative oxidation of adrenaline, catalysed by a conglomeration of different types of oxidases, inherent to a tissue like the auricle, cannot be simple or regular.

In the systems studied by us, we have found that ephedrine effectively protects adrenaline from oxidation by amine oxidase, thereby confirming the findings of Blaschko *et al.* (*loc. cit.*). These authors sought to explain the inhibiting effect of ephedrine by assuming an affinity of this compound for this enzyme. It is known that compounds having a configuration very nearly similar to that of the

specific substrate do inhibit enzyme activity, either competitively or non-competitively, in the former case being dependent on substrate concentration. On these grounds, Blaschko *et al.* correctly ascribe the protective effect of ephedrine on the system adrenaline-amine-oxidase to competitive inhibition. On the contrary,

GRAPH 3.
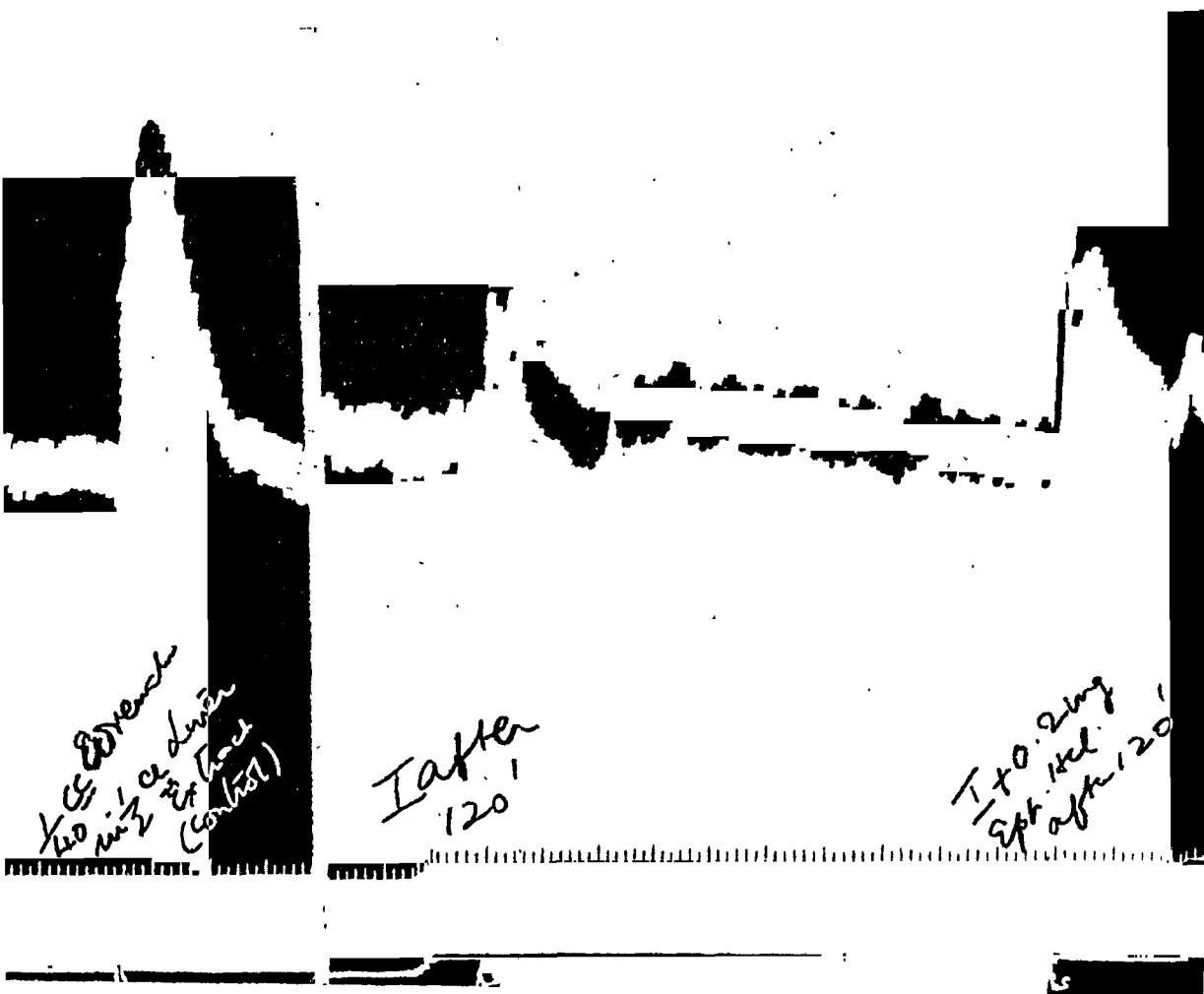
(*a*)   (*b*)

Dog 6·25 kg.—Chloretone (6–9–1939).
    (*a*) 0·025 mg. adrenaline in half c.c. liver extract (blood pressure).
    (*b*) I.   Control—blood pressure after 120 minutes.
       II.   Control + 0·2 mg. ephedrine in half c.c. liver extract after 120 minutes.

Gaddum describes the same effect as a case of substrate competition, although it has been shown definitely that ephedrine by itself is not a substrate for amine oxidase. If it is granted that the term 'substrate competition' is correctly

applicable to cases where more than one substrate compete for an enzyme, then the action of ephedrine we are dealing with falls out of this category.

It is also possible that ephedrine might act by ways other than enzyme inhibition, as suggested by Gaddum. Earlier, Burn (1932) thought ephedrine acted by liberating adrenaline from a local store, which became depleted unless re-filled by circulating adrenaline. The extent of protection, whatever be its nature, afforded by ephedrine to adrenaline must be limited. Unlike as *in vitro* studies, where we are dealing with individual enzymes, in the organism, comprising a complex enzyme system, adrenaline would be exposed to drastic oxidation against which ephedrine would have little power. The action of a complex system of enzymes can be offset only by a corresponding system of inhibitors. If then, adrenaline is to be effectively protected in the organism, besides ephedrine, other defensive mechanisms like that of ascorbic acid, inherently present or extraneously introduced, are essential.

SUMMARY.

1. Some of the facts and theories regarding the oxidation of adrenaline *in vitro* and *in vivo* have been reviewed.

2. The rôle of phenolases in the oxidation of adrenaline and the inhibition of their action by stabilizers, namely, HCN and ascorbic acid, have been studied. Ephedrine also appears to act as one such stabilizer although in a limited degree.

3. Further experimental evidence has been obtained to prove the inhibiting effect of ephedrine on the oxidation of the system adrenaline-amine-oxidase.

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ANÆMIA IN PREGNANCY IN INDIA : THE PRESENT POSITION.

BY

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ABOUT thirteen years ago, special attention was drawn to the importance of anæmia as a cause of maternal morbidity and of maternal and infantile mortality in India. During the intervening years a considerable amount of investigation has been undertaken both under the auspices of the Indian Research Fund Association and by independent workers. We feel that the time has now come to summarize the results of this work, to state the present position, and to indicate the general trend of these investigations.

MATERNAL MORTALITY IN INDIA.

The exceptionally high maternal mortality in child-birth in India may be looked upon as the circumstance that made an inquiry on this subject an urgent necessity.

Dr. Margaret Balfour who had retired from the Women Medical Service returned to this country at her own expense to investigate this subject. She received a grant for additional help from the Indian Research Fund Association.

She (Balfour, 1927b) collected data from Indian hospitals and found a maternal mortality rate of 21·5 per mille (244 deaths in 11,343 deliveries).

The registration of both births and deaths in India generally is unsatisfactory, and, as there is no special mechanism by which deaths associated with child-bearing are returned separately, the central or provincial public health reports give no indication of the true state of affairs.

In Bombay, however, where it was thought that the registration was comparatively accurate, the death rate was given as 16·7 per mille births in 1924 and in the Calcutta Corporation Health Officer's report for the same year it was given as 18 per mille.

In 1933, Sir John Megaw collected figures from rural dispensary doctors in different parts of India and gives a figure of 24·5 maternal deaths per 1,000 live

births. In a special inquiry in Calcutta, Neal Edwards (1939) found a similar rate and two inquiries in Madras obtained figures of 16·6 and 18·5 per 1,000 births. In Assam, as a result of a questionnaire, Balfour obtained an average figure of 42 per 1,000, and in a single area the extraordinary figure of 137 maternal deaths per 1,000 live births. These figures have to be compared with 3·8 and 3·16 per 1,000 live births in England and Wales in 1936 and 1937, respectively. It is unnecessary to emphasize this point further.

The part played by anæmia in this high mortality.

Balfour in her early investigations and personal experience in Bombay and Assam repeatedly expressed the opinion that about half the deaths were associated with anæmia.

She (Balfour, 1927a) placed anæmia second to osteomalacia as a cause of maternal morbidity and gave the figure as 27·6 per mille of pregnancies. Neal Edwards collected data from women's hospitals in India and found even higher figures for anæmia, 49·5 and 42·9 per thousand in 1936 and 1935, respectively. Napier and Das Gupta (1937b) found 158 per thousand pregnant coolie women in Assam definitely anæmic (below 50 per cent). Choudhury and Mangalik (1938) found 5·6 per cent of pregnant women in Agra ' anæmic '; in their published series of 41 cases they include two with 50 per cent hæmoglobin (6·87 g.) and seven over this amount.

Balfour (1927b) reported that 61·9 per cent of all maternal deaths in Bombay and 35·6 per cent of those from India generally were due to anæmia.

Mudaliar and Rao (1932) in Madras city found that 50 out of 436, or 11·5 per cent, of maternal deaths were due to anæmia, but Neal Edwards in her Calcutta inquiry (1936-37) found that 23·33 per cent of deaths were due to anæmia and from an analysis of 39 women's hospitals all over India, 18·00 per cent. Mitra (1933) gives the mortality due to anæmia during 1931 at a large maternity hospital in Calcutta as 47·9 per cent of the total maternal mortality.

The death rate in pregnancy anæmia.—The significance of the figures quoted below is limited, as the death rate will depend, firstly, on the severity of the cases admitted and, secondly, on the treatment given.

Number of patients.	Percentage of deaths.	Authority.
150	42	Balfour (1927a).
43	34·9	McSwiney (1927).
50	30	Wills and Mehta (1930a).
165	31·5	Mitra (1937).
561	36·5	do. (1937).

These figures do however show that the maternal mortality in pregnancy anæmia may be alarmingly high. Napier and Majumdar (1938) had eight deaths in a series of 58 cases (13·8 per cent), but there were in this series seven additional cases noted as 'failures' and it is probable that some of these patients died later in their homes. It is possible that a similar explanation may be given for Chatterjee's (1938) low figure—12 per cent.

An even higher infantile mortality is reported by all workers; for example, McSwiney (*loc. cit.*) gives a figure of 58·8 per cent.

Large as the figures are, they do not convey a true picture of the effect of anæmia, because amongst the cases of sepsis, which heads the list of causes of death, there are many instances in which, if the patients had not been severely anæmic as well, they would have recovered.

Whereas in Bombay and Calcutta sepsis and eclampsia, which appear above and below anæmia, as the first and third causes of death, respectively, occur in all countries, anæmia is usually classed as a negligible factor in Western countries, accounting for only 0·05 (England and Wales) and 0·08 (Scotland) per cent of maternal deaths. Anæmia is therefore not only very important as a cause of maternal mortality, but it is an outstanding one in this country as compared with other countries.

Further points which might be ascertained are the comparative importance of anæmia in Northern India and in any place where hookworm and/or malaria are uncommon.

Epidemiological data.

The early papers on the anæmia of pregnancy discuss the epidemiology, but usually give impressions rather than figures, and such figures as are given seldom include the composition of the populations and are thus valueless. Balfour (1927*a*), however, shows that in Bombay anæmia in pregnancy is more common in Mohammedans than in Hindus. The incidence per 1,000 is 93·8 and 40·1, respectively. However, osteomalacia and eclampsia, two other diseases which she includes in her table, show a far greater preponderance amongst Mohammedans, which suggest that the community is unhealthier generally. She states that they live on a better diet than the Hindus, but does not give any data for this observation.

Gupta (1932), reporting maternity cases admitted into the Eden Hospital, Calcutta, during 1928–1930, gave the total incidence of anæmia as 7·19 per cent; Hindus were 8·89 per cent, Mohammedans 19·09 per cent, and Indian Christians 22·54 per cent, the low total incidence being due to dilution with Europeans, none of whom were anæmic of 73 admitted, and with Anglo-Indians in whom the incidence was also low.

Choudhury and Mangalik (*loc. cit.*) found little difference in the communities in Agra: they give Hindus as 5·1 per cent and Mohammedans as 6·3 per cent.

There are very few data regarding the economic status of patients. Both Balfour and Wills repeatedly state that anæmia of pregnancy is not confined to

the poorer classes and that it is higher amongst some of the better-class communities. They produce no convincing figures to prove this. On the other hand, most of the data presented are from hospitals in which the poorer class of patient predominates, and, though anæmia is known to be far from uncommon amongst the ' private-patient ' class, comparable figures are not available. As far as Bombay is concerned the statement is probably dependent largely on the observation that anæmia in pregnancy is not common amongst a certain class of mill worker. The dietary surveys in Bombay (Wills and Talpade, 1930; Talpade, 1931) amongst different classes of patient, including the mill workers, produced mainly evidence of a negative nature, in that they showed that the individuals of all the classes surveyed lived on an ill-balanced diet; the diet amongst the mill workers was very deficient in fat and protein, particularly animal protein, and in vitamins A and C, but was not deficient in vitamin B; this did not, however, differ significantly from that of the pregnancy anæmics of the hospital class.

Seasonal incidence.—Balfour (1927a) in Bombay found the highest incidence from September to March. McSwiney (*loc. cit.*) and Mitra (1931, 1937) from July to December in Calcutta (*see* Table I).

TABLE I.

	Balfour (1927a) (percentage).	Mitra (1931).	McSwiney (1927).
January	10	4	3
February	7	2	2
March	13	1	1
April	5	5	0
May	2	3	0
June	1	6	4
July	5	8	5
August	6	6	3
September	10	11	7
October	9	12	3
November	14	10	7
December	18	18	8
TOTALS	100	86	43

Chatterjee (*loc. cit.*) gives the percentage incidence of pregnancy anæmia compared with total obstetric admissions at a Calcutta hospital over a 5-year period;

he shows that there is a marked preponderance from October to January with comparatively high percentages in September and February.

Mudaliar and Rao (*loc. cit.*) in Madras were only reporting on a small number. but show the highest incidence in June. Choudhury and Mangalik (*loc. cit.*) in Agra do not find any seasonal incidence. They point out that the pregnancy incidence increases in the second half of the year. This was shown also by Balfour in Bombay and is true in Calcutta, but in neither city is this increase in the anæmia incidence in the last four months of the year explainable on the greater number of pregnancies in these months. Balfour found diarrhœa more common in the second half of the year and considers that the anæmia incidence is correlated with this.

Age.—The question of age is too closely associated with that of parity to provide any significant information independently.

Parity.—Balfour (1927a). McSwiney (*loc. cit.*), and Chatterjee (*loc. cit.*) give the following figures for parity (Table II):—

TABLE II.

Authority :—	Balfour (1927a).	McSwiney (1927).	Chatterjee (1938).
Locality :—	Bombay.	Calcutta.	Calcutta.
Primiparæ ..	29	13	10
2nd pregnancy ..	22	10	7
3rd	13	6	10
4th	7	5	9
5th	9	2	7
6th	3	3	3
7th. 8th. or over ..	14	2	3
TOTALS ..	97	43	50

Mitra (1937) and Choudhury and Mangalik (*loc. cit.*) show graphs suggesting the same steadily declining incidence through the pregnancies. The value of these figures is reduced by the writers' failures to give the parity distribution amongst normal pregnancies. This will obviously vary in different populations. Mitra (1937) goes further and makes the misleading statement: 'Figure 2 shows the greater incidence in multiparæ (80 per cent) than in primiparæ'. Napier and Gupta (1937a) remedy this defect. but they are obviously dealing with an unusual population for the peak of the normal distribution curve is at the 3rd pregnancy :

in their figures the percentage of anæmia is 28·5 for primiparæ and this falls to 15·4, 13·3 and 12·2 in the succeeding pregnancies.

From the data analysed there appears to be evidence of a higher relative incidence of anæmia amongst primiparæ, but it is possible that in some instances this apparent predominance is due to the fact that women are more ready to come into hospital for their first pregnancy.

Prematurity.—Not very much is to be learnt from the month of pregnancy at which the patient first comes under observation as this may have little relationship to the time of onset of the anæmia. In nearly all the series reported, the incidence of anæmia rises towards the end of pregnancy, and very few cases are less than six months' pregnant when they first come under observation.

Discussion.—Studies of the anæmia of pregnancy in which an attempt is made to find epidemiological factors are usually based on the premise that there is one specific condition with one common cause. Discrepancies in the findings of different observers may well be explained on the grounds that there are multiple causes and that these are not equally represented in the various series of different observers. Discrepancies therefore assume a special importance and, further, it is obvious that we ought to obtain more data from different places in India and from different classes of patients, in order to decide more definitely whether the cause is single or multiple, and to correlate the various observations with the special local conditions, e.g., the incidence of various diseases, such as hookworm and malaria, and with the diet of the populations concerned.

Associated clinical picture.

Wills and Mehta (1930a) have stated that 16 of their 66 cases have been excluded on the grounds of malaria, hookworm infection, syphilis, or definite nephritis. The rest they claim are 'idiopathic' anæmia of pregnancy. This procedure of excluding certain cases seems scarcely justifiable and only likely to confuse the issue.

Fever.—This is a very common associated condition. Balfour (1927a) noted that the onset of the anæmia was with fever in exactly half her cases and that 83·3 per cent had fever at some time or another during the disease. Mitra (1931) reported fever in 36·7 per cent of cases, and Chatterjee (*loc. cit.*) in 90·6 per cent. Other workers refer to the frequency of fever. Napier found in his Assam cases that fever at some time during pregnancy was the rule in almost all cases.

Little importance can be attached to the percentage malarial parasite findings in the various series reported, as much will depend on the thoroughness of the search made. Balfour (1927a) reported 8·66 per cent plasmodium infections in her series, a high figure in a place where the general malarial incidence is not particularly high.

Splenic enlargement.—In Bombay, Balfour (1927a) reports 18 per cent splenic enlargement, McSwiney (*loc. cit.*) 23 per cent, Mitra (1931) 40·7 per cent, and Gupta (*loc. cit.*) 8·8 per cent in Calcutta; the last is probably too low a figure. In Assam, Napier and Bilimoria (1937) differentiated between the hæmatological groups;

.they reported 33 per cent enlarged spleens amongst hyperchromic cases, 14 per cent amongst hypochromic, and six per cent amongst non-anæmic pregnant women. Napier and Majumdar (*loc. cit.*), in a more malarious part of Assam, found 65 per cent of the anæmic pregnant women had enlarged spleens, against 30 per cent non-anæmic, if all degrees of enlargement were considered, and 43 per cent and 8 per cent, respectively, if definite measurable enlargement only was considered ; they also found that there was a ' significant ' difference between the iron-reacting and the liver-reacting groups, the latter showing a much higher incidence of splenic enlargement.

In Agra, Choudhury and Mangalik (*loc. cit.*) found that 55·6 per cent of the patients in their macrocytic group had enlarged spleens, but none of the rest.

All reports thus tend to emphasize a very high incidence of splenic hypertrophy amongst cases of anæmia in pregnancy.

Œdema.—All workers report a high incidence of œdema and many say it is a cent per cent finding.

Albumin in the urine.—Most workers report this in a large percentage of cases. Wills and Mehta (1930*a*) report albumin in the urine in 35 per cent of their cases, Balfour (1927*a*) in 48·6 per cent, McSwiney (*loc. cit.*) in 60·5 per cent, Mitra (1931) 26 per cent, and Gupta (*loc. cit.*) 25 per cent, but Chatterjee (*loc. cit.*) only gives 7·5 per cent ; this last figure can probably be ignored.

Gastro-intestinal symptoms.—Diarrhœa is a very common association ; it was reported in 38 per cent of Balfour's (1927*a*) series, in 44 per cent and 20·6 per cent of Mitra's (1931, 1937) two series, and in 74·1 per cent of the macrocytic group in Choudhury and Mangalik's (*loc. cit.*) series. In the Assam cases diarrhœa or dysentery occurred in at least two-thirds of the cases either shortly before or during the time that they were under observation. Balfour was so impressed with the frequency of the association that she attributed the higher incidence of anæmia in the second half of the year to the much higher incidence of diarrhœa and dysentery in Bombay at this season.

Balfour (1927*a*) reported vomiting (other than the ordinary vomiting associated with early pregnancy) in 40 per cent and sore tongue in 31 per cent of cases. Mitra (1931) mentions that 82·5 per cent of his series had a sore tongue. We have found in Calcutta and Assam this condition very hard to assess ; most of the women eat *pan* which disguises the appearance of the mouth and most of them have pyorrhœa, and it is difficult to elicit the information without a leading question ; our impression is that it is not a very prominent symptom. Choudhury and Mangalik (*loc. cit.*) state definitely that they have not noted it in their cases. Others do not mention either vomiting or sore mouth as common.

Gastric acidity is discussed later.

Wassermann and Kahn reactions.—McSwiney (*loc. cit.*) found a positive Wassermann reaction in 40 per cent of his cases. Balfour (1927*a*) reported that, in 15 out of 32 cases tested, the Kahn reaction was positive. Mitra (1931) found four out of 11 cases Wassermann positive ; Napier and Majumdar (*loc. cit.*) found the Wassermann reaction negative in 27 out of 36 cases, doubtful in three, and positive in six ;

most of the positive findings were in the hypochromic group. Chatterjee (*loc. cit.*) however found *none* of his cases positive, and other workers have not found more positives than the usual percentage of the general population. This subject requires further investigation as no large series has been done.

Treatment.—No one has described the treatment of anæmia in pregnancy with much confidence and those who have attempted any scientific appraisal of their results have usually approached the subject with the object of throwing light on the ætiology.

McSwiney (*loc. cit.*) advised treatment on general hygienic lines, with dietary, iron and arsenic by mouth or injection, small injections of whole blood, and the termination of pregnancy. He advised against blood transfusions.

Green-Armytage (1928) thought that the anæmia of pregnancy was a toxæmia and advocated the termination of pregnancy.

Wills and Mehta (1930*a*) used liver extract with good results.

Wills (1931) first drew attention to the value of Marmite. In a large number of her Bombay cases recovery followed the administration of 30 grammes of Marmite daily. The improvement occurred after a typical reticulocyte response.

Gupta (*loc. cit.*) recommended treatment on similar lines but included liver injections and Marmite. He noted that large transfusions caused abortion.

Mudaliar and Rao (*loc. cit.*) obtain no response with Marmite. This is possibly because many of their cases were hypochromic and probably iron-deficient; liver extract and iron produced the best response.

Mitra (1937) advocated all known forms of therapy but was vague as to the results obtained. He reports a high mortality. He seems to favour small repeated transfusions, but his remarks suggest theory rather than practice. He attributes good results to intravenous glucose. He concluded 'Cure is never vouchsafed unless pregnancy terminates'.

Choudhury and Mangalik (*loc. cit.*) also found Marmite useless. They obtained good results with Neo-Hepatex and Campolon in macrocytic cases, and with iron in microcytic. Transfusion was not used by them as they considered it unnecessary.

In Napier and Majumdar's (*loc. cit.*) series, treatment was experimental and usually one hæmatinic only was given.

Iron (in microcytic cases) or Marmite (in normocytic cases) given early seemed to be effective, but given in the later stages of pregnancy it had no effect until the uterus was empty. Campolon produced a more rapid, and a more certain, response in normo- or macrocytic cases, but again the inhibitory effect of the fœtus was marked, and, in the later stages of pregnancy, response was seldom satisfactory until the uterus was empty.

Transfusion seemed to have no specific effect but tended to cause abortion which was often followed by improvement.

Napier *et al.* (1938) found Anahæmin less effective than crude liver extract. This observation was confirmed by Wills and Evans (1938).

Hæmatological investigations.

The necessity for investigating the hæmatological picture of anæmia of pregnancy led to work in a number of divergent directions, which it will be necessary to consider separately. The first may be called the introduction and standardization of hæmatological methods and the obtaining of normal standards for Indian populations. The second is the attempt to separate into different hæmatological groups, in the hope that this may lead to an ætiological classification, the anæmias that occur (a) amongst the general population, both men and women, and (b) in pregnant women. A third direction has been the evolution and study of a new pathological and clinical syndrome, tropical macrocytic anæmia.

It will be convenient to discuss the last line of investigation first.

Tropical macrocytic anæmia.—Wills in her first paper showed very clearly that there was amongst her cases of anæmia of pregnancy a hyperchromic anæmia which was quite distinct from Addisonian pernicious anæmia, but which responded with a characteristic reticulocytosis to liver extract; she also described a similar disease in non-pregnant women. Her biochemical and clinical investigations in these cases did not suggest that the condition was a toxæmia of pregnancy, and on the grounds of some preliminary dietary investigations amongst her patients she suggested that it was most probably a dietary disease and she favoured deficiency of vitamins A and C, as being the probable cause (Wills and Mehta, 1930a).

A dietary survey amongst different Bombay communities seemed to add support to the suggestion that vitamin-A and -C deficiencies were the causative factors, as it was in the communities in which these deficiencies were most pronounced that most cases of this anæmia were discovered (Wills and Talpade, *loc. cit.*). More support came from animal experiments in which she showed that rats fed on diets deficient in vitamins A and C died of *Bartonella* anæmia (Wills and Mehta, 1930b). She then turned to monkeys as her experimental animals. She produced a macrocytic anæmia in monkeys by feeding them on a diet of polished rice, white bread, and chapattis, a more or less vitamin-free diet; to this she added vitamins A and C but without result; she then gave Marmite, with the result that the monkey's red cell count and its weight immediately improved (Wills and Bilimoria, 1932). She followed this up by an immediate clinical trial in Bombay and established the fact that Marmite would cure one form of anæmia of pregnancy, a macrocytic anæmia, that appeared to be most prevalent in Bombay (Wills, 1931).

This work of Wills had clearly established the fact that there was a macrocytic anæmia occurring amongst pregnant and non-pregnant women which was curable by some substance contained in autolysed yeast; this disease which was a new syndrome she called tropical macrocytic anæmia.

The subsequent work of Wills (1934) has not been done under the ægis of the Indian Research Fund Association; it is however important work and some of it has been done in India. Wills and her co-workers have shown that the actual substance that produces the improvement in this macrocytic anæmia is not vitamin B_1, B_4, lactoflavin, nicotinic acid, or vitamin B_6. She showed that, in experimental

monkeys fed on a diet deficient in vitamin-B complex, the West and Dakin fraction of liver extract (represented by Anahæmin) was not curative, but that the soluble fraction, after saturation with ammonium sulphate, of Campolon produced a reticulocytosis and cure. Napier (1938) confirmed this in the case of tropical macrocytic anæmia in Calcutta as did Wills and Evans (*loc. cit.*) in Bombay a few months later. Later, however, the former worker showed that in some cases a cure was effected with massive doses of Anahæmin, in conjunction with a good hospital diet (Napier, 1939b). Later, Foy and Kondi (1939) made the same observation with regard to macrocytic anæmia in Macedonia.

The present position is that the actual identity of the substance that brings about a cure in Wills' macrocytic anæmia has not been discovered, but a large number of substances have been excluded; to those substances mentioned above must be added Castle's extrinsic factor, as refined liver extracts (e.g., Anahæmin) which are curative in true pernicious anæmia are not as effective in this condition. We know that the substance is present in an ordinary mixed diet, and in a more concentrated form in autolysed yeast and in the cruder liver extracts.

In the experimental work with monkeys at present being undertaken by the writer with the assistance of Dr. Majumdar, we have so far failed to reproduce the Wills' macrocytic anæmia in our monkeys, though we have been able to show that the recovery time in monkeys subjected to repeated small bleedings was much longer when the monkeys were kept on a vitamin-B-deficient diet than when they were kept on a balanced diet.

Whilst it is a matter of more than academic interest to identify this factor, the writer does not feel that it is an absolutely essential piece of information, not, at any rate, to the extent that lack of this knowledge seriously prevents our obtaining a very much better understanding of the ætiology of the anæmias of pregnancy in India. We know that a diet deficient in vitamin-B complex will probably be deficient in this substance and tend to produce a particular type of macrocytic anæmia, and we know that autolysed yeast and most of the crude liver extracts contain it and, in uncomplicated cases of this anæmia, will effect a cure. The writer does not think that at this stage further experimental work in India directed solely towards identifying this factor will be profitable. It is highly technical work which is already being done in England by Wills and probably other workers. Later, it might be necessary to confirm her findings in India as we did in the case of her Anahæmin work.

We know that deficiency of a certain food factor will produce this particular macrocytic anæmia. We know also that many of the people who suffer from it live on a diet which is deficient in many essential substances, but none of the dietetic survey work so far done, either in Bombay or elsewhere, has been able to show that the diets associated with this anæmia were definitely deficient in vitamin-B complex, though it was seldom in excess. Wills has taken the view that the pregnant women's requirements are slightly in excess of those of a non-pregnant woman and that this deficiency is thereby exaggerated; she points out quite correctly that this same anæmia occurs in non-pregnant women and in men, but less frequently. In this connection the example of iron deficiency and hypochromic iron-deficiency

anæmia in pregnant women has been quoted. However, it has not been shown that the demands of the fœtus for vitamin-B complex are greater than for any other food substance, as is the case with iron which the fœtus stores in its liver in large quantities. All workers are not prepared to accept this comparatively simple explanation of the ætiology of the syndrome. Reference will be made to this point later.

Tropical macrocytic anæmia hæmolytica.—Napier (in 1936) drew attention to the fact that all macrocytic anæmias of tropical countries that responded to Marmite and liver extract were not Wills' tropical macrocytic anæmia; he pointed out that there was a hæmolytic group, associated with hyperbilirubinæmia, a high reticulocyte count, and usually splenic enlargement, possibly of malarial origin. Fairley and his co-workers (1938) found the same state of affairs in Macedonia; Fairley considered that there were two forms of tropical macrocytic anæmia, a hæmolytic and a non-hæmolytic. Napier (1939*b*) developed this idea further; he concluded that there was strong evidence in favour of the existence of a hæmopoietic principle, distinct from the hæmopoietic principle absent in pernicious anæmia and in no way associated with gastric dysfunction, which substance was essential to the proper maturation of the red cells; it was a substance that was not synthesized in the body and was not a residue of normal hæmolysis, but had to be provided in the food; its deficiency might be actual, that is, a true food deficiency, or relative, that is, due to the extra demands of excessive hæmolysis (e.g., of post-malarial reticulo-endothelial tissue hypertrophy), or due to malabsorption, of, for example, long-continued diarrhœa.

These observations of Napier and Fairley and their co-workers have not so much introduced a new clinical and pathological syndrome, as they have broadened the basis of Wills' syndrome, so that it includes more than one type of anæmia, and they have certainly widened the ætiological possibilities.

Normal hæmatological standards for Indian populations.

One of the first difficulties encountered by any one attempting to work on anæmias in India ten years ago was the complete lack of any normal standards for Indian populations, and the primitive state of hæmatology in India. At this time most of the large hospitals in the country depended on the Tallqvist scale for the estimation of hæmoglobin; this and the differential count were practically the only routine hæmatological investigations ever carried out. This lack of precision in hæmatological methods was not confined to India and the fact that the 100-per-cent mark in some hæmoglobinometers indicated a hæmoglobin content of 13·8 grammes in 100 c.c. of blood, whereas in others it corresponded to 17·2 grammes showed that hæmatology throughout the world was in a very confused state at this time.

At one meeting of medical officers that was called in Assam to discuss with Balfour her proposals for investigating the anæmia of pregnancy, a great deal of time was spent on discussing whether 60 per cent or 65 per cent should be looked upon as the normal for Indian women; no consideration was given to the scale or

the method of measurement used, which was of course usually the Tallqvist. This is not quoted as a criticism of the medical officers present, but to demonstrate the confused thinking that was prevalent at the time.

At the 7th Research Workers' Conference, Mehta made a strong plea for the collection of hæmatological data from normal Indian populations. A very important achievement of the investigations under the Indian Research Fund Association has been the stimulation of work of this kind, much of which has actually been done by officers employed by the association. Papers in which the different hæmatological methods were critically examined were written by Sokhey, Napier, and others, and have undoubtedly helped considerably in uniformalizing hæmatological methods in India. Some of the findings amongst normal populations are given here.

Normal hæmatological data.

In the three tables below some of the important data on hæmatological standards for Indian populations have been collected. In the United States and in Great Britain much work on normal standards has been done; we have quoted only representative data from reliable authorities.

Red cells.—There is a striking uniformity in the findings. The average count for males in Western countries is 5·5 millions, in Indian towns 5·4 millions, and in coolie populations 5·3 millions. For females, the average for Western countries is 4·8 millions and for Indian populations 4·5 millions.

A series by Basu and Chatterjee (1937) have been excluded as they are so grossly at variance with the findings of other workers; they give, for a series of women in Calcutta, 3·6 millions with a mean deviation (*sic*) of 0·49 million.

Hæmoglobin.—There is less uniformity in the hæmoglobin estimations; for men the means given range from 14·5 grammes to 16 grammes in Western countries (higher and lower readings are given by other authorities not quoted here) and those for men in Indian towns cover about the same range. On the other hand, the figures obtained amongst coolie populations are markedly lower (12 to 13 grammes) and show high coefficients of variation. Similarly amongst the women, 13·5 grammes is about the average for Western countries and 13 grammes for Indian towns, against 10·5 grammes for coolie women.

Hæmatological values.—This constancy in the red cell counts and deficiency in the hæmoglobin levels amongst coolies is naturally reflected in the corpuscular values, and both the mean corpuscular volume (MCV) and the mean corpuscular hæmoglobin (MCH) of the cells of the coolie populations is in each case much lower than that of the town dwellers. It will be noted that the MCV of the two Cachar series were not nearly as low as those of the two Assam series; it will be noted also that the mean corpuscular hæmoglobin concentration (MCHC), (which is complementary to the MCV, if the MCH is constant) is exceptionally low in the former series. These two interdependent observations can probably be accounted for by the unsatisfactory working of the centrifuge which was noted in the report on the Cachar series.

TABLE III.

Normal red cell counts.

Sex.	Ages.	Locality.	Economic status.	Number.	Mean red cells per c.mm. in millions.	Standard deviation.	Authority.
Males ..	25—45	Calcutta	Mixed	50	5·362	± 0·633	Napier and Das Gupta (1935b).
,, ..	20—45	,,	,,	30	5·533	± 0·490	do. (1936).
,, ..	19—30	Bombay	Students, etc.	121	5·110	± 0·380	Sokhey et al. (1937).
,, ..	19—30	Assam	Coolies	24	5·353	± 0·620	Napier and Das Gupta (1935c).
,, ..	19—30	,,	,,	20	5·270	± 0·710	do. (1936).
,, ..	..	Cachar	,,	25	5·057	± 0·563	Napier and Majumdar (1938).
,, ..	Adults	U.S.A.	..	..	5·400	..	Castle and Minot (1936).
,, ..	,,	Britain	..	..	5·690	..	Whitby and Britton (1939).
,, ..	,,	,,	..	..	5·428	..	Price-Jones (1931).
Females, non-pregnant ..	14—38	Calcutta	..	125	4·615	± 0·409	Napier (1939a).
,, ..	16—30	Bombay	Middle class	101	4·470	± 0·330	Sokhey et al. (1938).
,, ..	17—30	Delhi	,,	101	4·560	± 0·250	Benjamin (1939).
,, ..	..	Assam	Coolies	20	4·550	± 0·650	Napier and Bilimoria (1937).
,, ..	..	Cachar	,,	25	4·454	± 0·705	Napier and Majumdar (1938).
,, ..	18—22	Michigan	Students	50	4·750	..	Bethel (1936).
,, ..	..	Britain	..	..	4·800	..	Whitby and Britton (1939).
,, ..	..	,,	..	..	5·012	..	Price-Jones (1931).
Females, pregnant ..	..	Assam	Coolies	40	4·650	± 0·620	Napier and Bilimoria (1937).
,, ..	..	Michigan	,,	28	4·120	..	Bethel (1936).

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TABLE IV.

Normal hæmoglobin levels.

Sex.	Ages.	Locality.	Economic status.	Hæmoglobin in grammes per 100 c.c.	Standard deviation.	Number on which based.	Authority.
Males	19—30	Bombay	Students	15·37	± 0·96	121	Sokhey et al. (1937).
,,	25—45	Calcutta	Mixed	14·77	± 1·36	50	Napier and Das Gupta (1935b).
,,	25—45	,,	,,	15·70	± 0·91	30	do. (1936).
,,	Adults	Assam	Coolies	12·63	± 1·41	20	do. (1936).
,,	,,	Cachar	,,	12·60	± 1·83	25	Napier and Majumdar (1938).
,,	,,	Assam	,,	11·83	± 1·67	24	Napier and Das Gupta (1935c).
,,	,,	Shivrajpur	,,	13·74	± 1·79	47	Sen (Napier, 1939a).
,,	..	..	..	12·95	± 1·72	49	do.
,,	Adults	U. S. A.	..	16·00	..	..	Castle and Minot (1936).
,,	,,	Britain	..	15·60	..	..	Whitby and Britton (1939).
Females	..	..	..	14·50	..	..	Price-Jones (1931).
,,	18—30	Bombay	..	12·99	± 1·10	101	Sokhey et al. (1938).
,,	14—38	Calcutta	..	12·63	± 1·01	128	Napier (1939a).
,,	17—30	Delhi	Middle class	13·11	± 0·81	100	Benjamin (1939).
,,	..	Coonoor (6,000 feet).	..	15·81	± 2·54	100	Radhakrishna Rao (1938).
,,	..	Cachar	Coolies	10·40	± 1·74	25	Napier and Majumdar (1938).
,,	..	Assam	,,	10·80	± 2·30	20	Napier and Bilimoria (1937).
,,	..	Britain	..	13·60	..	..	Price-Jones (1931).
,,	..	,,	..	13·70	..	..	Whitby and Britton (1939).
,,	18—22	Michigan	Students	13·76	..	50	Bethel (1936).
Pregnant women	..	Assam	Coolies	10·70	± 1·60	40	Napier and Bilimoria (1937).
,,	..	,,	(Obvious anæmics excluded).	9·22	..	228	Napier and Das Gupta (1937b).
,,	..	,,	..	9·99	± 1·72	192	do. (1937b).
,,	..	Michigan	..	11·85	..	28	Bethel (1936).
,,	..	Coonoor (6,000 feet).	Ante-natal clinic (mixed)	15·52	± 2·52	100	Radhakrishna Rao (1938).

TABLE V.

Hæmatological values.

Sex.	Locality.	Number.	Mean corpuscular volume.	Mean corpuscular haemoglobin.	Mean corpuscular haemoglobin concentration.	Authority.
Males ..	Calcutta	30	90·49	28·53	31·07	Napier and Das Gupta (1936).
,, ..	Bombay	121	87·08*	30·01*	34·54*	Sokhey *et al.* (1937).
,, ..	Assam	24	71·29	23·93	32·50	Napier and Das Gupta (1936).
,, ..	Cachar	25	84·93	25·14	29·72	Napier and Majumdar (1938).
Unspecified {	U. S. A.	..	87	27·5	35	Castle and Minot (1936).
	Britain	..	86	29·5	34	Whitby and Britton (1939).
Females ..	Calcutta	128	86·82	27·42	31·57	Napier (1939a).
,, ..	Bombay	101	88·53*	29·06*	32·86*	Sokhey *et al.* (1938).
,, ..	Delhi	100	85·64*	28·76*	33·58*	Benjamin (1939).
,, ..	Assam	20	77·30	24·50	31·20	Napier and Bilimoria (1937).
,, ..	Cachar	25	82·49	23·42	28·67	Napier and Majumdar (1938).
,, ..	Michigan	50	86·3	..	..	Bethel (1936).
Pregnant females ..	Assam	40	72·10	23·80	32·60	Napier and Bilimoria (1937).
,, ..	Michigan	28	92·00	..	..	Bethel (1936).

* Calculated from data given, after applying factor × 1·09 to cell volume to allow for shrinkage, where this has not been done.

Hyperbilirubinæmia.—The van den Bergh test was not done in many of the 'normal' series; the assumption was that it would be 'negative'. Vaughan and Hazlewood (1938) did this test in a series of 'normal' individuals in England and obtained the results shown in Table VI. Data from Assam coolies are also shown :—

TABLE VI.

Van den Bergh indirect.

Locality.	Number of persons.	0·5 mg. or less per 100 c.c.	Less than 1·0 mg. but over 0·5.	1·0 mg. or over.	Percentage over 1·0 mg.	Authority.
England ..	100	64	32	6	6	Vaughan and Hazlewood (1938).
Cachar ..	50	39	6	5	10	Napier and Majumdar (1938).
Assam ..	40	33	3	4	10	Napier and Bilimoria (1937).

Vaughan and Hazlewood find a distinctly higher percentage of positives than many other English and American observers, but they are not as high as those amongst so-called normal individuals in Assam.

Reticulocytes.—The normal figure is about 0·5 per cent of the total red cells: some writers give a lower figure from the Indian 'normals', the following data are quoted (Table VII) :—

TABLE VII.

Reticulocytes.

Locality.	Material.	Number of persons.	Percentage.	Standard deviation.	Authority.
Calcutta ..	Men	50	0·67	± 0·37	Napier and Das Gupta (1935b).
,, ..	Women	122	0·37	± 0·27	Napier (1939a).
Assam ..	Men and women	41	2·17	± 1·92	Napier and Das Gupta (1935c).
,, ..	Pregnant women	40	2·10	+ 1·80	Napier and Bilimoria (1937).

This suggests that the normal reticulocyte count in Indians is the same as that in Europeans, and it seems probable that in the Assam series the high reticulocyte count is evidence of disturbed erythropoiesis.

Red-cell diameters.—The only reliable way of learning the mean diameter of red cells is by Price-Jones' method, or some slight modification of it. A few series have been done on normal Indians. Maplestone (Chaudhuri, 1933) found the mean diameter of 500 cells of each of ten healthy Indians to be 7.270μ. Sankaran and Radhakrishna Rao (1938) found a curiously low figure for Madrassi Indians; the mean of about 500 cells from each of 25 normal Indians, male and female, was $6.85\mu \pm 0.28\mu$; there was no significant difference between males and females in this series. Napier *et al.* on the other hand obtained a figure of $7.288\mu \pm 0.468\mu$ for 50 Bengal males. Price-Jones' figure was $7.202\mu \pm 0.172\mu$ for 100 healthy individuals in England.

The low figure obtained by Sankaran and Radhakrishna Rao is not explained but otherwise there seemed little indication that the measurements differ materially from those obtained elsewhere; Price-Jones' figure is representative of the European findings. The much wider range of variation amongst the so-called normals probably indicates that strict normality is less common in Calcutta than in London.

Discussion.—These data all conform to the conclusion that, as far as the red cell series is concerned, there is no physiological difference in the blood pictures between Europeans in temperate countries and Indians in India, and that differences, when they occur, are due to some pathological cause; this cause may be anything from a food deficiency to a sub-clinical infection. In the case of the coolie populations, there is in the hæmatological data further evidence to support this suggestion, in the high coefficient of variation in the hæmoglobin estimations, in the high mean reticulocyte count, and in the hyperbilirubinæmia that are commonly encountered amongst Assam coolies.

The question naturally arises as to the reason for this lower level of hæmoglobin amongst the Assam coolie populations and whether it can be traced to the special conditions prevailing in Assam. The only figures we can quote from our data are those from Shivrajpur coolies; these are economically comparable, but are employed in a different part of the country. The hæmoglobin means are much closer to those of the Assam coolies than to those of the city-dwelling Indians. This suggests that the hæmoglobin deficiency is one associated with economic status and dietary rather than with the particular local conditions. It has been our experience in Calcutta, as well as in Assam, that not only has the average healthy ryot a lower hæmoglobin level, but that his cells tend to be smaller and, though long-continued treatment with iron will bring his red cells up to the 5.5 millions level or even higher and his MCHC up to 35 per cent, on account of the persistently small size of his red cells his hæmoglobin will not rise to the normal level.

Napier and Das Gupta (1936) did a small experiment in this connection; they gave a course of iron treatment to six healthy coolies, and only managed to raise the mean hæmoglobin level from 11.2 grammes to 12.0 grammes. This is certainly

an experiment that should be repeated, but the implication of this very poor response to iron therapy is that there is some other factor operating which keeps down the size of the red cell and consequently the hæmoglobin level.

It will obviously be easier to answer this question when we have more data. We want 'normal' data for village populations in different parts of India where different conditions prevail. This is an important point, for one may safely conclude that there will be some connection between the general low level of hæmoglobin and the incidence of gross anæmia in any population, even if the cause is not exactly the same. Meanwhile, any further consideration of this aspect of the problem can be postponed until some of the possible causes of the anæmia are discussed.

The blood picture in pregnant women.

Dieckmann and Wegner (1934) and Bethel (1936) have found that there is a progressive increase in blood volume throughout pregnancy. This is not accompanied by a corresponding increase in cells, so that there is a relative decrease in the number of cells in a fixed volume of blood—this physiological anæmia amounts to about a 14-per cent deficiency, i.e., 14 per cent below the normal level for non-pregnant women. In order to make allowances for the natural variations in individual hæmoglobin percentages these writers allow a further margin, and state that any reading below 10 grammes should be considered as indicating anæmia —this figure is about 27 per cent below Bethel's mean for non-pregnant women (13·78 grammes). Translating this into terms of Indian populations, we get about 9·5 grammes for town and 7·7 grammes for coolie populations on the 13-gramme and 10·5-gramme basis, respectively.

Practically all investigators in India have considered figures mostly well below these levels, so there is little chance that any have been dealing with a purely physiological anæmia.

In the only comparative series quoted (Napier and Bilimoria, *loc. cit.*) there is no evidence of this physiological anæmia, but there is a possible fallacy here, in that, as investigations were being conducted in the anæmia of pregnancy at that time, it is probable that the anæmic pregnant women had been combed out from the pregnant series much more carefully than the non-pregnant anæmics from the other control series; this suggestion is supported by the much higher standard deviation in the non-pregnant series. Napier and Das Gupta (1937b) found a distinctly lower level of hæmoglobin amongst pregnant coolies, but noted that when the obviously anæmic women had been excluded the mean was much the same as amongst non-pregnant 'normal' women; they concluded that, whilst evidence of a general lowering of the hæmoglobin level was absent, there was a much higher percentage of gross anæmia amongst the pregnant women.

Where one is dealing with a much higher percentage of pathological anæmia in a population, it is going to be more difficult to recognize the physiological anæmia, but it is a point on which further investigation might be carried out. Meanwhile, it is reasonable to assume that the factors which produce this physiological anæmia in America also operate in India.

Bethel (*loc. cit.*) in America found that the size of the cell tended to increase throughout pregnancy reaching its maximum about the 36th week when the hæmoglobin is at its lowest level. He found a mean figure of 86·3 cu.μ for non-pregnant women and 92·0 cu.μ for pregnant women in the 3rd trimester. The only comparable figures in India do not indicate that this occurs here : Napier and Bilimoria (*loc. cit.*) give figures of 77·3 and 72·1 for non-pregnant and pregnant women, respectively. These figures are obtained, it will be seen, in a population in which there is general microcytosis : it is possible that the change in cell volume is an indication of the natural exaggeration during pregnancy of the dominant deficiency ; in the Assam group this is an iron deficiency. This is certainly a point on which further work should be done.

Gastric acidity.—This has a double association with anæmia, in that a low gastric acidity has been associated with deficient absorption of iron—though recent work (Moore et al., 1937) does not confirm this—and that free acid is completely absent in true Addisonian pernicious anæmia. Strauss and Castle (1933) pointed out that gastric secretion was often depressed in pregnancy and it has been suggested that this might be a cause of the iron-deficiency anæmia in this state. Bethel (*loc. cit.*) did not find any relationship between gastric secretion and hæmoglobin level in pregnant women.

Wills and Mehta (1930a) in a series of 25 pregnant anæmic women found three with achlorhydria, one with hypochlorhydria, and two with hyperchlorhydria : the rest were within normal limits.

There is no evidence that the gastric acidity curve in Indians differs from that of Europeans (Napier and Das Gupta, 1935d ; Napier, Chaudhuri and Rai Chaudhuri, 1938 ; Rao, 1937). Napier and Das Gupta (1937a) found, in two series of about 40 cases each, the gastric acidity in anæmic coolies slightly lower than that of normal Indians. Achlorhydria was relatively uncommon ; it was found in five cases of the first series (gruel meal without histamin), and in the second series (alcohol test meal) in only one case and in that case at a second examination histamin caused secretion of free acid.

Napier and Majumdar (*loc. cit.*) did fractional gastric analyses on 41 anæmic pregnant women. The majority (26) were isochlorhydric, six were hypochlorhydric and three others achlorhydric (without histamin). Analysed differentially, they showed a lower acid level in the group that responded to liver therapy.

Choudhury and Mangalik (*loc. cit.*) did a gastric analysis in 18 pregnant anæmic cases and found achlorhydria in three and hypochlorhydria in five. Mitra (1937) found achlorhydria in one out of six cases tested, and Chatterjee (Chatterjee and Basu, 1939) 'a low acid curve' in four out of 15 cases. Mudaliar and Rao (*loc. cit.*) provide the only exceptional series and they found four cases of achlorhydria (without histamin) and five of hypochlorhydria in ten cases tested.

There is little suggestion in the data here summarized that there is any connection between gastric acidity and the anæmia of pregnant women in India.

Hæmatological surveys of the anæmias that occur in Indian populations.

It is very surprising that, although anæmia is probably second only to fever in the list of most commonly-occurring symptoms in any Indian hospital or dispensary, no attempt was made to study anæmia *per se* until a few years ago. This makes the task of reviewing this work comparatively easy. We have not combed the literature for reports on individual cases, or small groups of cases, such as that of Mackie (1929) who read a paper at a meeting of a Medical Society in 1907 on 'malignant anæmia of the tropics'; the paper nearly suffered the fate of the majority of papers read at medical meetings in India and would have been forgotten had its author not found a copy of the typescript and published a résumé of it in 1929. But we found no studies of a larger series of anæmic patients in which any attempt had been made at hæmatological or ætiological classification. This is not altogether surprising in view of the backward state of hæmatology, in India in particular and in the world in general, to which reference has already been made.

Anæmia amongst the general population.—As a preliminary measure to studying the anæmia of pregnancy in the same population, Napier and Das Gupta (1937*a*) investigated the anæmia amongst the general coolie population of a group of tea estates in Assam. Blood examinations before and after treatment were carried out in 100 cases of severe anæmia; these investigations were carried out during two successive cold weathers and the two series are analysed separately. In the first series there were 58 coolies, men and women; as the cell volume estimations were not considered satisfactory, the cases were classified according to the hæmoglobin content of the cells, i.e., the MCH. The vast majority were markedly hypochromic; in 53 cases the MCH was below $28 \cdot 5\gamma\gamma$, the mean of the city-dwelling Indian 'normals', and 45 were below $22\gamma\gamma$, the mean of the 'normal' coolie. Two cases only, with MCH's of $33\gamma\gamma$, might be considered to be hyperchromic. The cases were arbitrarily divided into two groups, definitely hypochromic, i.e., below $20\gamma\gamma$, and orthochromic, above $20\gamma\gamma$.

After analysing the data, clinical and pathological, in these two groups, separately, the writers concluded that it was doubtful if they could be considered as two separate hæmatological groups, though the orthochromic group showed a special tendency to hyperbilirubinæmia, with which splenic enlargement was correlated, and achlorhydria, and did not respond so well to treatment with anthelmintics and iron. There were more females in the orthochromic group.

The second series was even more markedly hypochromic; there were only two cases above $22\gamma\gamma$. The MCV is also reported in this series; two cases would be considered macrocytic judged by some standards, being above 100 cu.μ, but the mean of the series was 69·7 cu.μ and the large majority were below 80 cu.μ. They were therefore not divided, but considered as one group. In this investigation emphasis was laid on treatment and frequent blood examinations were done throughout the course of treatment. Also an attempt was made to gauge the effect of slightly different procedures, including a dietetic experiment.

Eight coolies were kept on a well-balanced (relatively) high-calorie diet for four weeks; there was no improvement in the hæmoglobin level but there was

improvement in the clinical picture, including loss of œdema. They were then given large doses of iron (27 grammes of ferrous sulphate daily) for three weeks and in four weeks the mean hæmoglobin of the group had risen from four to ten grammes. In the whole series of 41 cases (the 42nd patient absconded), 39 showed definite improvement on iron administration. Though diet *per se* had no effect on the anæmia, the groups in which the diet was most satisfactory showed the best response to the iron treatment. However, in very few cases did the hæmoglobin level improve beyond the level which had been found to be 'normal' in this coolie population.

It appears to have been shown beyond dispute that the dominant defect, as far as the hæmopoietic system is concerned, in this community is a hypochromic microcytic iron-deficiency anæmia. The cause of this anæmia is not so definite; a hundred per cent of the coolies had a hookworm infection, most of them a heavy infection, but no correlation between the hookworm load and the level of hæmoglobin can be shown. The diet of the coolie is low in iron, but it is uncertain if it is actually deficient; this point will be discussed later. In the absence of any gastric-acid deficiency, which might have led to malabsorption, it is reasonable to conclude that this anæmia is due to the continual drain, by blood loss through hookworm infection, on the iron reserves in a population on low, if not actually deficient, iron intake. Macdonald (1939) questions this conclusion because of the lack of correlation between the hookworm load and the degree of the anæmia. But the cent-per-cent hookworm infection cannot be denied, the methods of estimating hookworm load are open to considerable error, and finally it is only claimed that it is *one* of the factors concerned.

The writers (Napier and Das Gupta, 1937a) considered that there was evidence of some other limiting factor which prevents the blood cell returning to its normal size, as in this series the red cells increased to their normal number and the hæmoglobin concentration in each cell, the MCHC, reached the normal level, but, on account of the small size of the cell, it could not contain its full complement of hæmoglobin, so that the MCH and the hæmoglobin per cent only reached the lower, so-called normal, levels of this coolie population; this limiting factor is thus operating not only in the frankly anæmic coolies but in the whole population.

It will be apparent that in this population the anæmia of any particular group, such as the anæmia of pregnant women, must be considered against this background of widespread hypochromic microcytic anæmia and of a general lower level of cell size and hæmoglobin in the whole population. It must also be clear that unless this is done a very wrong impression might be obtained of the nature of such anæmia. No other similar surveys of anæmia amongst the general population in other areas have been reported.

The material for such an analysis with reference to the hospital out-patient population in Calcutta is available, and this analysis will be made. This is certainly a line of work that might well be undertaken in a number of places, but, as in the case of other survey work, when once a lead has been given, it should not be considered as a legitimate charge on funds ear-marked for research. It is a suitable line of work for medical units at colleges or universities.

Anæmia amongst pregnant women.—Balfour (1927a) collected hæmatological data in 25 cases of her series. There are no details as to how these cases were selected. She gives the means only of the 25 observations, so that one has no idea of their distribution, but, as she gives 1·4 as the mean colour index, one may assume that the majority were cases of hyperchromic anæmia. McSwiney (*loc. cit.*) makes no attempt to analyse his data, but from his case notes it is apparent that there are both hyperchromic and hypochromic cases in his series.

Wills and Mehta (1930a) vitiate their series, as a series, by the exclusion of 16 of their original 66 cases on the grounds that they were suffering from malaria, hookworm infection, syphilis, or definite nephritis ; they have classed the remainder as 'idiopathic'. Both the propriety and the possibility of excluding such cases is questioned. The essential difference between the study and the treatment of a case in a sanitarily advanced country on the one hand and in a sanitarily backward country, such as India, on the other is that in the latter one has always to make one's study against a background of widespread infections, such as malaria and hookworm, and of malnutrition, both general and special. So in an attempt to trace the ætiological factor or factors, of a clinical syndrome, one cannot say ' this infection, or this food deficiency, is common to almost the whole population, whereas the syndrome occurs only in a few, therefore, we can dismiss it as a possible ætiological factor'. Each infection and each food deficiency must be considered as possible contributing factors whose interactions may produce the syndrome with or without the addition of some metabolic toxæmia, and/or of some physiological stress, such as pregnancy. It would, for example, in the case of pregnancy anæmia, be absurd to exclude all cases showing ankylostome infections, for in the Assam series this infection occurred in every case almost without exception. Similarly, every woman (in this series) has had malaria at some time during her life, usually both recently and frequently. Further, the possibility of excluding all cases in which the malaria causes the anæmia is doubted, because a single blood examination is not sufficient to exclude even present infections and it is frequent past infections that are as likely to produce anæmia.

Wills and Mehta class their remaining 50 cases as ' idiopathic ' and consider them as a single group. From a hæmatological point of view they are not, however, a homogeneous group ; the colour indices in the 50 cases varies from 0·7 to 1·8 ; in 15 it is below 1, in nine from 1 to 1·09 and in 26 cases above this, but in only nine is the colour index 1·40 or more.

Mudaliar and Rao (*loc. cit.*) also treated their cases as a single group. The colour indices suggest that they are not ; of 32 cases seven were below 1·0, 14 between 1 and 1·09, and 11 were above this, of which only one was above 1·30. These last workers failed to obtain the same results with Marmite in the treatment of pregnancy anæmia that Wills had reported. This is not surprising, as it is obvious that there are fewer truly hyperchromic cases in their series.

Whilst it is reasonable to assume that all anæmias in pregnancy have not the same ætiology, it is difficult to decide how the cases should be divided up. Wills and Mehta's procedure of excluding all cases in which certain other infections are evident is an attempt to group cases, but is unsatisfactory for the reasons given

above. Mitra (1937) however developed this idea; by a more elaborate process of selection he excluded 103 of his 165 cases, leaving 62 idiopathic cases. Nothing in his subsequent analysis of these groups seems to justify this division, except possibly the lower mortality in the 'idiopathic' group. In view of the hæmatological findings this writer reports (e.g., 52 macrocytic cases of which 30 were hypochromic) his choice of a clinical classification was wise, and, though the result was a negative one, as such, it has some value.

Gupta (*loc. cit.*) classified his cases according to severity, using the red cell count as his criterion—below one million, between one and two millions, etc. There are obvious limitations to such a classification, though it may be usefully adopted for primary grouping when there are large numbers of cases.

The majority of workers in India have adopted some hæmatological classification, either according to the size or the hæmoglobin content of the cell, or both. This method is criticized by Chatterjee and Basu (1938) who claim that during the development of th's anæmia the size of the red cell goes through a regular cycle of stages; commencing as normocytic the anæmia becomes microcytic, then if the case deteriorates it passes through a normocytic hyperchromic stage and becomes macrocytic, or conversely if it improves it changes back to normocytic. To explain these strange blood findings they invent a pathological cycle in the bone marrow in which there are successive stages of regeneration, compensation and bone marrow failure. They give examples of cases in which these changes are said to have occurred; the meagre data only show that in the particular cases—errors of technique being excepted—the blood picture changes from one stage to the next, but proof that a regular cycle occurs in any number of cases is entirely lacking.

The same idea of cyclical changes in the red cells has been entertained by Hare (1939) who considers that there is evidence that the size and the hæmoglobin content of the red cells change according to the stage of the pregnancy. He cites six cases in which the cell size tended to decrease between the 28th and the 36th week, increasing again towards the end of pregnancy or post partum. He points out that the 28th to 36th week is the period of maximum growth of the fœtus. It is interesting that he shows the exact reverse of the course of events that Bethel (*vide supra*) reports in normal pregnancy; Bethel showed that the size of the cell tended to increase throughout the second half of pregnancy reaching its maximum about the 36th week after which it tended to decrease again. Hare's observation, if the changes in these six cases can be accepted as the usual change in the population with which he is dealing, supports the suggestion already made above that a dominant deficiency is exaggerated at this stage of pregnancy by the demands of the fœtus.

Napier and Bilimoria (*loc. cit.*), working amongst the same population in which Napier and Das Gupta (1937a) had made a survey of the anæmias of the general population (*vide supra*), divided the cases into groups according to their MCH and hæmoglobin level. Comparing these two groupings, the non-pregnant and the pregnant, it is clear that in the latter series some new factor comes into operation. The hypochromic anæmias are still in the majority, but there is a hyperchromic group, which has been subdivided into two groups according to the severity of

anæmia. The less anæmic group, the writers have suggested, might be considered as physiological; this is in keeping with the suggestions made above in which the arbitrary figure of 7·7 grammes, based on Dieckmann and Wegner's (*loc. cit.*) observations, was chosen for coolie populations, and with Bethel's (*loc. cit.*) observations regarding the increase in size of the cell during pregnancy. Amongst the other points that arose in this analysis was the fact that the hypochromic cases tended to come under observation at an earlier stage in the pregnancy than the hyperchromic cases who practically all reported first in the last trimester; the suggestion is that in the latter group the anæmia was precipitated or aggravated by the pregnancy, whereas in the other the pregnancy was an incident in the course of the anæmia. The significantly higher incidence of splenic enlargement in the hyperchromic group has already been referred to, and there was also a 'very significant' preponderance of hyperbilirubinæmia in the hyperchromic groups, as compared with the hypochromic group; in fact, 16 out of 18 of the former group showed a positive indirect van den Bergh reaction, against less than half of the latter. In this series there is a strong association between hookworm infection and the hypochromic group, but not between hookworm infection and the other groups or the controls.

Napier and Majumdar (*loc. cit.*) were working in another district in Assam. Investigations amongst normal coolies had shown that the normal blood picture was of much the same order as had been found in other coolie populations, though the cell size seemed to be larger. (This was to some extent explained by the inadequacy of the electric current in this district leading to incomplete 'packing' in the cell volume estimations). No survey of the anæmias of the general population was undertaken here. Hookworm infection was almost a hundred per cent in incidence, but the infections were not so intense as in the other area; on the other hand this was a more malarious district.

The first classification of the cases was, as before, according to the hæmoglobin content of the cells MCH, but, as there was a central hospital in this district, it was possible to follow the results of treatment and later a re-classification of the cases was made according to the effect of treatment, into an iron-responding group and a liver-Marmite-responding group. In this series the initial hæmoglobin level was in no case above 7·7 grammes (*vide supra*). As there were few truly hyperchromic cases in this series, the cases were put into two groups with the dividing line at $22\gamma\gamma$; this divided them into two almost equal groups. Thus, the mean MCH of this series was slightly but distinctly higher than that of the previous group if only the definitely anæmic cases are considered. In the re-classification little change was effected; deaths and failures were excluded and three borderline cases on either side were changed over, otherwise the hypochromic cases became the iron-responding and the orthochromic-hyperchromic the liver-Marmite-responding group. Analysis of the rest of the data according to the two groups showed the high correlation between enlarged spleen and response to liver or Marmite treatment, which has already been referred to, and a very much higher incidence of hyperbilirubinæmia in this same group; thus, there was a positive van den Bergh in 10 per cent of the controls, in 25 per cent of the iron group and in 53 per cent

of the liver-Marmite group. There was on the other hand no difference in the hookworm infection between the two groups and a very slightly greater tendency towards a low acidity in the liver-Marmite group.

Sternal punctures were done in a number of cases; the majority showed a normoblastic hyperplasia, and in no case was there the typical megaloblastic hyperplasia that one sees in pernicious anæmia, but in a few cases there were a few megaloblasts and a high percentage of erythroblasts.

A point that was brought out in this investigation was the depressing effect of the fœtus in the later stages of pregnancy on the hæmopoietic response to treatment; this was more noticeable in the liver-Marmite group. When the treatment was given early in pregnancy or after parturition response was usually immediate, but when it was given in the last months of pregnancy there was often a slight reticulocyte response, indicating some specific action, with a very slight upward movement of the hæmoglobin curve or only maintenance of the previous level, until after parturition when there was an immediate sharp rise.

An attempt was made in the first of these inquiries to ascertain the diet of the individual patients, but the method was not found satisfactory, and it was decided that more information could be obtained by dietary surveys amongst the population from which they came. A survey was subsequently carried out in each of the two areas in which these hæmatological investigations have been carried out. One of these surveys was reported by Wilson and Mitra (1938). The two surveys produced materially the same results :—

The total calories were only a little below the standard usually considered necessary for Indian workers, but were mainly derived from carbohydrates : the total protein intake was low and the animal protein almost a negligible figure. Total fat was also very low, below 12 grammes, and most of it was of vegetable origin. Of the minerals, calcium was exceptionally low, being only about one-sixth of the usual requirements; the figure for iron was low, though adequate if it was all in available form, but there was some doubt on this point. Of the vitamins, the intakes of A and B complex were low, though apparently not actually deficient judged on most standards, but the vitamin-C intake was very low and, as this was mainly derived from vegetables that were cooked, saturation with this vitamin was improbable.

Mitra (1939) re-examined the question of iron intake in the light of later work on the availability of iron and concluded that these people were living on a deficient iron intake. This question of the availability of iron is, however, a controversial one, and whilst it is not possible to accept Mitra's conclusions, it is apparent that people of the poorer classes whose main diet is rice are living on a low available iron intake and that the more this rice is milled the lower will be this intake.

Macdonald (*loc. cit.*), in a separate investigation amongst the same population in which the first of these two inquiries was carried out, found a 'very significantly' lower hæmoglobin level amongst coolies living on milled rice as compared with those taking home-pounded rice.

Choudhury and Mangalik (*loc. cit.*) divided their cases into three groups, macrocytic (MCV over 90 cu.μ and over 29 $\gamma\gamma$), microcytic (below these figures), and mixed (MCV above 90 cu.μ and MCH below 29 $\gamma\gamma$). There were 27 macrocytic, six microcytic, and eight mixed cases. The indirect van den Bergh was positive in 74·1 per cent of the macrocytic group and in 28·6 per cent of the rest. The MCHC was below 30 per cent in all the hypochromic and mixed cases, and above this in nearly all the macrocytic cases. The high percentage of splenic enlargement in the macrocytic group and its absence in the others has already been referred to.

Conclusion.—The two hæmatological surveys in Assam appear to show that, superimposed on the hypochromic anæmia of the general population, there is hyperchromic anæmia which is associated with the later months of pregnancy. There is also evidence of the association of malaria with the hyperchromic anæmia. The whole population lives on an ill-balanced diet deficient in a number of elements; it seems probable that the hypochromic anæmia can be accounted for by the low intake of available iron and the widespread hookworm infection. Whilst a poor diet and malaria infection are common to the whole community, there is little evidence of hyperchromic anæmia in the general population; it is possible however that a ' slight tendency ' to hyperchromic anæmia may be disguised by the marked iron deficiency (this one knows from experience does occur), only to be brought out as a frank anæmia by the extra demands of pregnancy, or by a toxæmia of pregnancy, which may be a *conditioned* toxæmia, in that it would not occur in a person protected by an adequate diet.

These two surveys have also emphasized the importance of knowing the background against which a disease is being studied. When Balfour first went to Assam to start these investigations she obtained a number of blood films from cases of pregnancy anæmia and sent them to Wills who was working in Bombay. Wills was naturally cautious in arriving at any conclusion from such inadequate material, but she expressed considerable doubts as to whether the same problem existed in Assam as in Bombay, because most of the slides showed a definitely microcytic picture. She was quite right in expressing this opinion on the evidence she had before her, but the data here presented suggests that there is a macrocytic anæmia in Assam associated with pregnancy, and it is probably the same syndrome as is found in Bombay.

The hæmatological picture in the Agra cases (Choudhury and Mangalik, *loc. cit.*) is entirely different; here we have a predominantly macrocytic hyperchromic anæmia, apparently undisguised by any hypochromic tendency in the general population, it is unfortunate that there has been no survey of the anæmia in this population to compare it with. The association of malaria is again suggested by the high incidence of splenic enlargement and hyperbilirubinæmia.

The importance of such surveys in different localities where different ætiological factors prevail must also be apparent. From the inquiry which was recently carried out in Calcutta under Neal Edwards and Napier, similar data will be available when the analysis of the records is complete, but from other places in India, particularly rural districts, practically no information is available.

SUMMARY AND CONCLUSIONS.

1. There are good reasons for believing that a high maternal mortality is the rule throughout India. Special investigations have provided figures from 16·6 to the appalling, and probably exceptional, figure of 138 maternal deaths per 1,000 live births, compared with 3·16 per cent in the whole of England and Wales in 1937.

2. Anæmia occupies a high place in the lists of causes of maternal morbidity and mortality in this country; in many places it is second and in some it is first, and, as a contributory cause of death, it is probably even more important than these lists indicate.

3. As in many Western countries anæmia is a negligible cause of maternal mortality, it is obvious that there are special conditions prevalent in India which do not exist, or which exist to a much less extent, in these other countries.

4. The incidence of this anæmia appears to be highest in the second half of the year, even after allowance is made for the greater number of pregnancies at this time. The anæmia usually first attracts medical attention in the third trimester, and the incidence is apparently slightly higher amongst primiparæ.

5. Œdema occurs in nearly all cases of pregnancy anæmia, and fever in more than half; albumin is found in the urine in 25 to 50 per cent of cases, and diarrhœa occurs in a similar percentage. A relatively high incidence of splenic enlargement is reported by most workers, particularly amongst macrocytic cases, and some have noted a higher incidence of Wassermann-positive cases than occurs amongst the general population.

6. It has been shown that the blood picture of the normal healthy city-dwelling Indian living on a good mixed diet conforms closely to the standards that are based on observations made amongst Europeans in Europe and America, but, on the other hand, that in rural populations and in Indians of the lower economic strata, the hæmoglobin level of the whole population may be much lower than the above-mentioned standards and there may be other evidence in the blood picture of dys-hæmopoiesis; whilst these observations apply also to the so-called normal individuals, there are in the latter populations a large number who show definite clinical signs of anæmia.

7. With rare exceptions, the ætiological factors, in so far as they have been identified, that produce clinical anæmia, whilst they exert their greatest effect in the case of the frankly anæmic individual, also affect the individuals of the general population to a greater or lesser extent, and it seems reasonable to assume that these are the factors mainly responsible for producing the state of dys-hæmopoiesis widespread in these particular populations.

8. It has been shown that amongst the general populations in places where the anæmia has been studied there are three types of anæmia that are very common. These are :—

(a) A microcytic hypochromic iron-deficiency anæmia due to living on a diet actually deficient in available iron, or to continuous loss of blood,

usually from hookworm infection, in people living on a diet low in available iron.

(b) A macrocytic hyperchromic anæmia due to a deficiency in the diet of some substance that is present in autolysed yeast and in crude liver extracts, which anæmia is distinguishable from pernicious anæmia by the presence of free acid in the gastric secretions, the absence of neurological symptoms, a negative van den Bergh (indirect) test, and the absence of typical response to purified liver extracts (Anahæmin).

(c) A second type of macrocytic hyperchromic anæmia, distinguishable from the other type by evidence of increased hæmolysis—namely, hyperbilirubinæmia (a positive van den Bergh reaction) and a high reticulocyte percentage—and usually splenomegaly, which is probably associated with chronic malaria *and* some degree of dietary deficiency.

9. It is generally recognized that there is normally in the later months of pregnancy an anæmia, which may be a purely physiological anæmia, or due to a relative dietary (? protein) deficiency and which is common in many communities in other countries ; with this anæmia is associated an increase in the mean corpuscular volume of the red cells.

10. The incidence of anæmia in pregnancy in India appears to be correlated with that of the anæmia in the general population, but in pregnancy the incidence is higher and the condition assumes greater importance.

11. In pregnancy the anæmia shows a more marked macrocytic tendency than the anæmia in the general population, though in some localities this is almost completely disguised by the associated iron deficiency (or other microcytic-anæmia-producing factor).

12. Anæmia in pregnancy is amenable to suitable treatment in the early months of pregnancy, and in some cases in the later months but in the majority of these it is only possible to stop or slow down the deterioration of the blood picture until after parturition, when an immediate and rapid improvement commences in practically all except those cases complicated by sepsis. This sudden and rapid improvement is comparable to the sharp rise in hæmoglobin that occurs in certain cases of malaria when the infection is controlled by efficient treatment ; this suggests that the inhibitory factor is of the nature of a toxæmia associated with the actual presence of the fœtus.

13. The evidence so far collected appears to point to the fact that anæmia in pregnancy is due to a combination of the ætiological factors that produce anæmia in the general population with some factor associated with pregnancy itself and the presence of the fœtus.

Some of the common ætiological factors are known, probably others are still to be discovered : of those that are known, the most important are hookworm infection and a low available iron intake, as the cause of microcytic anæmia, and specific and general dietary deficiency (e.g., absence of the specific factor that occurs in autolysed yeast and of biologically-valuable protein), alone or in combination with

chronic malarial infection, with its associated splenomegaly, reticulo-endothelial proliferation, and increased blood destruction, as the causes of macrocytic anæmia.

These factors, together with the specific pregnancy factor, vary in their influence in different populations that have been investigated and in different individuals, and produce a variety of blood pictures microcytic and hypochromic, macrocytic and hyperchromic, and, where two opposing influences are acting more or less equally in the same individual, normocytic and hypochromic, macrocytic or normocytic and orthochromic, or rarely some other combination.

The effect of pregnancy itself, it has been suggested, is solely in the extra demands of that inexorable parasite, the fœtus, of essential food substance ; this suggestion is supported by the fact that in the sub-clinical anæmia of pregnancy the peak of the blood dyscrasia is correlated with the period of maximum growth of the fœtus. There is, however, in the writer's opinion evidence, in addition, of some toxæmia associated with the actual presence of the fœtus, which inhibits hæmopoietic response in severe forms of anæmia when in the later months of pregnancy the deficient substances are administered even in full doses, although, after the uterus has been emptied, there is an immediate maximal response ; the toxæmia is probably a ' conditioned toxæmia ' which only operates when there is an associated specific food deficiency, either absolute or relative.

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CLINICAL INVESTIGATIONS INTO ANÆMIA IN ASSAM.

PARTS I—VII.

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CONTENTS.

Part I.

GENERAL INTRODUCTION, MATERIAL, AND TECHNIQUE.

INTRODUCTION.

One of the commonest and most disabling conditions met with in tea-estate coolies in Assam is a severe anæmia. Macdonald (1939) gives it as his opinion that, among these coolies, ' the chief entity responsible for sickness and death is anæmia '. This anæmia has been shown by Napier and Das Gupta (1937a) to be, usually, of the microcytic and hypochromic type with the addition, chiefly among pregnant females, as has been shown by Napier and Majumdar (1938), of certain types having hyperchromic and macrocytic tendencies. Its ætiology is a matter of conjecture and some argument, though several possible ætiological factors are apparent to the tea-estate medical officer. It might be due to hookworm infection, to chronic malaria, to dietetic deficiencies or to residence, in strange conditions, in a foreign country, for all the coolies are non-indigenous

and the vast majority are not born in Assam. Macdonald (*loc. cit.*) has also postulated the consumption of certain types of rice and an unknown infective factor as being concerned in the ætiology of the disease. I have not been able, as yet, to test the validity of these theories on the population among whom my own work is being done.

Hæmoglobin standards for persons in normal health have been stated for Bombay by Sokhey *et al.* (1937, 1938), for Madras by Sankaran and Rajagopal (1938), and for Calcutta by Napier (1937). All these determinations show that the blood of Indians living in the three principal cities of India is, so far as hæmoglobin content is concerned, not inferior to the standards laid down for England by Price-Jones (1931) and for America by Haden (1933).

Hæmoglobin standards for tea-estate coolies in certain districts of Assam have also been determined by Napier (1937), Napier and Bilimoria (1937), and Napier and Majumdar (*loc. cit.*). The means of those observations are very markedly lower than any of the other observations quoted, indicating a partially anæmic state of the tea-estate coolie even when in good health. Unfortunately these observations appear to be the only ones so far made on rural populations and since those particular populations were non-indigenous, it would seem as though there are no existing hæmatological standards for rural India.

My position as medical officer of a group of tea estates has rendered it possible for me to study several of the problems concerning the ætiology of these anæmias in a population under a fair measure of control.

MATERIAL.

The human material dealt with in these investigations varied accordingly to the nature of the particular inquiry. Actually, investigations have been carried out on samples of almost every community resident on the estates. Members of the European staff with their wives and children were examined at one period. Members of the Indian staff and their wives and families, also, were examined. Other investigations were carried out on apparently healthy men, women, and children, of the coolie class, on newly recruited labourers, on clinically anæmic women who were not pregnant, and on pregnant women who were found to be anæmic.

TECHNIQUE.

As I was dealing with a similar community to that in which the majority of the work of Napier and his assistants was carried out and as much of my work has been complementary to theirs, I followed their technique, on general lines, as far as possible. The scope of each investigation varied according to the information required, but the items of technique were standardized as follows :—

Blood examination.

Hæmoglobin.—All hæmoglobin estimations were made with the Sahli instrument, except for certain large-scale surveys in which the Tallqvist scale was used.

The Sahli instrument was graduated in grammes per 100 c.c. of blood. This was chosen as it appears to be the most accurate of the hæmoglobinometers in general clinical use and the complete absence of extensive laboratory facilities prevented the use of such methods as those of van Slyke or Wong. The precaution was taken of using a new instrument to eliminate fading of the standard. In all cases the reading was taken after twenty minutes.

Red and white cells.—A Thoma-Zeiss hæmocytometer was used for the cell counts.

Corpuscular volume.—For the estimation of corpuscular volume a centrifuge doing 3,000 revolutions per minute was used and spinning continued until packing was complete. Readings were taken after thirteen minutes' spinning and again after another two minutes. Usually no further settlement had taken place but in the occasional case which did show a difference, a further two minutes' spinning showed that packing was complete in fifteen minutes. In all cases the value obtained was multiplied by Wintrobe's shrinkage factor, 1·09, and the figures quoted in this paper are the corrected figures. These examinations were carried out on oxalated blood, approximately 5 c.c. of venous blood being withdrawn into a test-tube containing 0·01 g. of potassium oxalate.

Abnormal red cells.—Search was made for abnormal red cells in thin films stained with Leishman.

The general plan of hæmatological technique was intended to reproduce as far as possible that standardized by Napier and his assistants and described by them in 'Hæmatological Studies in Indians', published at various times in the *Indian Journal of Medical Research.*

Stool examination.

Helminthic ova.—For the diagnosis of helminthic infection a saline concentration method was used. Approximately $\frac{1}{2}$ c.c. of the fæces was vigorously shaken up with 10 c.c. of saturated common salt solution and with this mixture a round glass-tube holding 10 c.c. was filled. A cover-slip was placed in contact with the surface of the fluid and allowed to remain for at least thirty minutes. Examination was carried out with a $\frac{2}{3}$-inch objective and a $\times$ 9 ocular and degrees of infection were expressed as follows :—

One egg in less than every five fields · ..	.. Heavy.
One egg in less than every twenty fields ..	.. Moderate.
One egg in more than every twenty fields	.. Light.

Urine examination.

Only the more common abnormalities were tested for.

Part II.

HÆMOGLOBIN STANDARDS IN DIFFERING SECTIONS OF THE POPULATION IN A GROUP OF TEA ESTATES.

INTRODUCTION.

It is plausible to suggest that the severe anæmia of tea-estate coolies is merely an exacerbation of the partial anæmia of that population already referred to and that the same ætiological factor is responsible for both conditions. This Part describes an experiment designed as an attempt to assess the relative importance of some of the more obvious factors.

For this investigation three groups were studied—a group of eighty coolie-class men, women, and children, drawn from one estate and all apparently in good health, selected at random on a family basis; the second group consisted of forty men, women, and children of the middle class. These were members of the Indian staff drawn from all the estates in my medical charge together with their wives and families; they were all in good health and were also selected on a family basis. The third group comprised all the European residents of the district available at the time who were in good health.

An estimation of the hæmoglobin content of the blood was made and a stool examination carried out on every person in all three groups. The possibility of the presence of dietetic deficiencies was dealt with by attempting to assess the standard of living. The coolie group was divided into three sub-groups whose daily expenditure on food was over one anna, one anna, and under one anna, *per capita.* Similarly, the Indian staff were divided into three sub-groups with daily *per capita* food expenditure of six annas or more, five annas, and four annas or less. It was not possible to sub-divide the European group who all live according to better class standards, spending about two rupees daily on food. All kinds of food are readily obtainable but, in the case of young bachelors, who are catered for by their native cooks, the quality of the food served may not always be commensurate with the cost to the consumer. In the virtual absence of kala-azar in this district the presence of splenomegaly was taken to be indicative of chronic malaria. Residence in Assam was dealt with by sub-dividing each group into those who had spent less than two years in the Province ('new residents') and the remainder ('old residents'). For each sub-group the mean hæmoglobin content was calculated and statistical tests were applied to determine whether such differences as were found between different groups and sub-groups were such as might have arisen by chance or not.

RESULTS.

The results of the survey as a whole are given in Table I and the hæmoglobin means, expressed as functions of the ætiological factors investigated, are shown for

coolies, Indian staff and European staff, respectively, in Tables II, III, and IV. In several instances in the latter two groups the sub-groups are too small to allow of reliable statistical comparison, so the principle of the smoothed mean has been introduced. The mean hæmoglobin contents of the whole coolie group and of the adult decades of that group are materially lower than the standards, 11·83 g. and 12·63 g. per 100 c.c. for males, and 10·03 g. and 11·30 g. per 100 c.c. for females, stated by Napier (1937) and than the figures, 12·60 g. and 10·40 g. per 100 c.c. for males and females respectively, determined by Napier and Majumdar (*loc. cit.*). Those estimations were done on coolies in the Jorehaut and Labac areas and I am given to understand that the standard of living in the Jorehaut area is somewhat higher than in my own and that the Labac area is more healthy than mine. The estate from which the coolies examined in this investigation came has an average annual death rate of about 28 per 1,000.

The figures for the Indian staff, also, are materially lower than any of the determinations made on city-living Indians by the workers referred to in Part I. I pointed this fact out to some of them and they, themselves, stated quite definitely, and on their own initiative, that their standard of living is inferior to that of other members of their own class in the large cities, the difference being chiefly due to the higher cost of food in the mofussil.

On the other hand, in the European group, if adult males are considered separately, their mean hæmoglobin content is found to be 14·87 g. per 100 c.c. which is higher than the figure quoted by Price-Jones (*loc. cit.*) for London (14·5 g.). Similarly, the mean for adult females in my series is 14·23 g. which is higher than Price-Jones' London figure (13·5 g.) and is also higher than the mean quoted by Haden (*loc. cit.*) for American women (12·93 g.). These findings suggest that there is some factor operative in Assam which causes a lowering of the hæmoglobin level in the Indian born but does not affect the white immigrant.

STATISTICAL ANALYSIS.

The standard deviation of the difference of two means can be readily calculated from the formula :—

$$\text{S.D. (difference)} = \sqrt{\frac{(\text{S.D.})^2}{N} + \frac{(\text{s.d.})^2}{n}}$$

where S.D. is the standard deviation of one mean and N is the number of observations on which it is based, and s.d. and n are the corresponding figures for the other mean. The standard error of the difference will be regarded, for the purposes of this study, as being equal to plus or minus twice the standard deviation and, therefore, any difference between two means which is greater than its own standard error is not one which might easily have arisen by chance, due to errors of sampling. Differences fulfilling this condition will be termed 'significant'.

Table I.—The differences between the three main groups are significant.

Table II.—The differences between sub-groups A and D and A and E are significant, as also is the difference between males and females. There is no significant difference between the 'dietetic' sub-groups nor between the 'hookworm infection' sections. The difference between those with splenomegaly and those without is barely significant and that between new and old residents is just significant.

Table III.—The differences between the age-groups A-B and B-C are both significant. The male-female difference is not significant and the means of the 'dietetic' sub-groups are very nearly identical. With regard to splenomegaly, the sub-group K is of small size so that, though the difference H-K is significant, too great importance must not be attached to it. In the 'hookworm infection' series, again, no difference is significant. No new residents were examined in this section of the population.

Table IV.—The only significant differences are those between the 'age' sub-groups A-B and A-C. Among those sections dealing with splenomegaly and hookworm infection, such correlation as was found is negative.

TABLE I.

Results of survey as a whole.

		Coolies.	Indian staff.	European staff.
Total number examined	.. ..	80	40	39
Number per cent with enlarged spleen	..	37·5	12·5	7·7
Number per cent infected with hookworm		70	32·5	41
Hæmoglobin in grammes per 100 c.c.	Highest observation ..	11·3	13·7	17·5
	Lowest observation ..	7·9	9·6	12·0
	Mean	10·04	12·54	14·45
	Standard deviation ..	0·79	0·88	1·18
Average daily expenditure *per capita* on food		One anna.	Five annas.	Two rupees.

TABLE II.

Details of survey of eighty coolies.

Sub-groups.			Number examined.	Mean hæmoglobin content.	Standard deviation.
Age in decades	0–9	A	12	9·62	0·49
	10–19	B	16	9·67	0·73
	20–29	C	20	10·10	0·93
	30–39	D	14	10·47	0·68
	40–49	E	11	10·35	0·68
	50–59	F	7	9·96	0·55
Sex	Male	G	43	10·40	0·76
	Female	H	37	9·60	0·61
Diet	Good	K	13	10·05	0·70
	Fair	L	41	10·13	0·79
	Poor	M	26	9·86	0·80
Splenomegaly	Absent	N	50	10·17	0·86
	Present	O	30	9·79	0·64
Hookworm infection.	Absent	P	24	10·16	0·75
	Present	R	56	9·97	0·80
	Light	S	33	10·05	0·86
	Moderate	T	8	9·92	0·51
	Heavy	W	15	9·84	0·75
Residence	New	X	9	10·60	0·69
	Old	Y	71	9·96	0·78

TABLE III.

Details of survey of forty Indian staff.

Sub-groups.		Number examined.	Mean hæmoglobin content.	Smoothed mean.	Standard deviation.
Age in decades	0–9 ..	11	13·04	12·95 A	0·67
	10–19 ..	3	12·59		
	20–29 ..	5	11·51	11·84 B	0·80
	30–39 ..	10	12·00		
	40–49 ..	7	12·82	12·97 C	0·67
	50–59 ..	4	13·21		
Sex ..	Male ..	28	12·58	D	0·72
	Female ..	12	12·42	E	1·17
Diet ..	Good ..	18	12·73	12·51 F	0·93
	Fair ..	8	12·00		
	Poor ..	14	12·58	G	0·76
Splenomegaly ..	Absent ..	35	12·62	H	0·76
	Present ..	5	11·55	K	1·12
Hookworm infection.	Absent ..	27	12·70	L	0·72
	Present ..	13	12·20	M	1·07
	Light ..	7	12·24	N	1·11
	Moderate	6	12·16	O	0·83
	Heavy ..	Nil	..	P	..
Residence ..	New ..	Nil	..	R	..
	Old ..	40	12·54	S	0·88

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TABLE IV.

Details of survey of thirty-nine European staff.

Sub-groups.		Number examined.	Mean hæmoglobin content.	Smoothed mean.	Standard deviation.
Age in decades	0–9	5	12·82	12·82 A	0·84
	10–19	*Nil*	..		
	20–29	7	13·83	14·57 B	1·17
	30–39	17	14·88		
	40–49	7	15·12	14·99 C	0·58
	50–59	3	14·69		
Sex	Male	27	14·68	D	1·26
	Female	12	13·94	E	0·89
Splenomegaly	Absent	36	14·39	F	1·19
	Present	3	15·23	G	0·52
Hookworm infection.	Absent	23	14·23	H	0·98
	Present	16	14·78	K	1·26
	Light	11	14·66	L	1·53
	Moderate	4	15·00	15·04 M	0·75
	Heavy	1	15·19		
Residence	New	10	14·24	N	1·06
	Old	29	14·54	O	1·08

CONCLUSIONS.

In the European group, as pointed out above, the hæmoglobin levels for adult males and females compare favourably with those determined for white races elsewhere. The mean for the decade 0–9, moreover, lies well within the normal limits for children as stated by Fitz-Hugh (1938) and there are no significant differences between any pairs of observations not connected with age grouping. I therefore conclude that the climate of Assam is probably not an important factor in the

production of anæmia and that, in this group, malaria and hookworm infection are equally lacking in effect.

Among the Indian staff, the hæmoglobin level in the children is slightly higher than that of the same age group in the European section and is, of course, well within Fitz-Hugh's normal limits. This occurs in spite of the fact that these children are constantly exposed to the ravages of malaria and hookworm infection. This fact, coupled with the absence of significant differences between the various hookworm infection sub-groups, lends additional support to the impression that neither malaria nor hookworm infection is the primary cause of anæmia in Assam. There is, however, a very striking and significant (statistically) lowering of the hæmoglobin level in the middle two decades of life and a return to childhood level in the fifth and sixth decades. As a fact which may have some bearing on this extraordinary state of affairs, I would remark that salaries and standard of living, generally, are highest among the older members of the Indian staff and that it is a point of honour with the average member of this class to feed his children as well as he can possibly afford, even denying himself to do so. Whatever it may be, there is certainly some factor operative in Assam which prevents the blood of the Indian born attaining normal levels in adult life and it would appear that this factor is not racial since it does not operate in the large cities of India.

We are now in a position to examine the figures in the coolie group in the light of these findings. Though the difference between old and new residents is just significant, too much reliance cannot be placed on this fact as the sub-group X is small. Hookworm infection does not appear to be of any greater moment as a primary ætiological factor than it does in the other two groups and, though chronic malaria, as indicated by splenomegaly, does appear to have some bearing, it must be borne in mind that, of the thirty persons with enlarged spleens, the majority were under the age of twenty. The group, as a whole, shows the normal rise in hæmoglobin from childhood up to adult life so that the lower levels of the first two decades are probably physiological and not, as might be argued, the result of weighing by chronic malaria. Within the rather narrow limits of the local coolie standard of living, variations in diet do not appear to be important. The significantly lower level of hæmoglobin in females is not surprising in view of the fact that the coolie's wife works on the estate in addition to bearing children and performing her household duties. The striking fact about this group, as a whole, is that, though the normal increase in hæmoglobin level occurs as adult life is reached (in contrast to what occurs in the Indian staff), the levels throughout are so definitely depressed. The obvious inference is that the factor responsible is operative throughout life. In order to test the validity of this assumption, I made weekly estimations in seven babies from the end of the first week of life to the end of the eighth. All these babies were in good health during the whole period of the investigation : they were all breast-fed and the mothers were healthy and were not receiving any special diet or any medical treatment. The means for each week together with Fitz-Hugh's normal ranges are set out in Table V. The coolie babies are seen to lose 4·28 grammes of hæmoglobin per 100 c.c. of blood during the first two months of life. The difference between the arithmetic means of Fitz-Hugh's

maxima and minima is exactly 4·0. It seems therefore reasonable to assume that, in the case of the coolies, the partially anæmic state is actually congenital and that, starting off with a low standard, subsequent variations in the hæmoglobin level conform to the ordinary physiological variations seen in the white races.

TABLE V.

Weekly observations on seven coolie babies.

Age in weeks.	Mean hæmoglobin content in g. per 100 c.c.	NORMAL HÆMOGLOBIN CONTENT IN G. PER 100 C.C.	
		Minimum.	Maximum.
One ..	14·34	17·0 arithmetic mean—22·5	28·0
Two ..	12·84		
Three ..	11·98		
Four ..	11·13		
Five ..	11·10	11·0 arithmetic mean—18·5	26·0
Six ..	10·48		
Seven ..	10·16		
Eight ...	10·06		

When one considers the enormously conglomerate character of the coolie population, drawn from many provinces and of many castes, the only factor, operating throughout life, which is likely to be common to all and will agree with the facts of the present inquiry, is the dietetic factor. Napier and Majumdar (*loc. cit.*) report that a diet survey, carried out among tea-estate coolies in Cachar, revealed a strikingly severe deficiency in calcium intake. That finding bears out my own clinical impression, gained in the course of my visits to the estates, that the intake of calcium in the form of milk is woefully insufficient. Only a limited amount of fresh cow's milk is available, at a somewhat excessive price, and practically all this is used by the European community and the Indian staff who give the majority of their supply to their children. The coolies hardly ever taste milk except when they become hospital in-patients. The lack of this particular item of diet may or may not be of primary importance as an ætiological factor but I feel convinced that some continuous dietetic insufficiency offers the only adequate explanation of the enormous differences seen in Table I and of the marked correlation between hæmoglobin level and expenditure on food, which is so close that it could hardly have arisen by chance.

SUMMARY.

1. Attention is drawn to the low hæmoglobin level of the blood of tea-estate coolies as compared with city-living Indians and it is suggested that the commonly-occurring severe microcytic hypochromic anæmia is an exacerbation of this partially anæmic state.

2. A survey is described which included estimation of hæmoglobin and examination of stools for hookworm ova in coolies, Indian staff, and European staff, associated with a group of tea estates. Details of the technique employed are given.

The results are tabulated and subjected to statistical analysis.

3. Within the scope of the investigation, the only constant and important correlation found to exist was that between standard of living and hæmoglobin content.

Part III.

POSSIBLE INDICATIONS OF THE HÆMATOLOGICAL STANDARDS OF THE RURAL POPULATIONS OF CERTAIN PARTS OF INDIA.

INTRODUCTION.

It has been pointed out in Part II of this paper that the blood of tea-estate coolies in Assam is deficient in hæmoglobin as compared with western and urban Indian standards and the conclusion was reached that this deficiency is congenital. Since none of these coolies are indigenous and only a small proportion are Assam-born it is difficult to see how residence in Assam can be blamed for it. The obvious implication is that the village dwellers of India, from whose ranks the tea-estate labourer is drawn, suffer from a similar deficiency of hæmoglobin : in other words, that the blood standards of rural India are not the same as those of urban India. Unfortunately, as was pointed out in Part I, no worker has yet recorded standards for rural India. This Part records an attempt to make good this deficiency.

Since every year, in the spring, a large number of rural Indians come up to Assam as recruited labourers for the tea estates, it appeared that here was a sample for investigation ready to hand. In order to avoid vitiating the results great care was taken to ensure that no person selected for examination had ever been in Assam before. All were carefully examined to exclude physical disease and all examinations were made within forty-eight hours of the subject's arrival on the estate, that is within a fortnight of his leaving his own home. By careful questioning, again, recruits from malarious villages and those who were subject to attacks of 'fever' were excluded and this precaution was extended further by examining only married couples who had children with them and none of whose children had any enlargement of the spleen.

I think it can fairly be claimed that the persons examined were reasonably free from disease and were, in fact, a picked sample of the tea-labour recruit population. There is no reason to suppose that they were not, also, a picked sample of the rural population from which they were drawn.

Eleven men and eleven women, fulfilling the conditions laid down above, were subjected to full hæmatological and stool examinations according to the technique described in Part I. All examinations were made between the hours of 2-0 p.m. and 4-0 p.m.

DISTRICTS OF ORIGIN.

The districts from which the subjects were recruited are marked with black discs on the Map.

12 persons came from Koraput.
4 ,, ,, ,, Ganjam.
2 ,, ,, ,, Sambalpur.
2 ,, ,, ,, Chaibassa.
2 ,, ,, ,, Ranchi.

MAP.

Sketch-map of India showing 'districts of origin'.
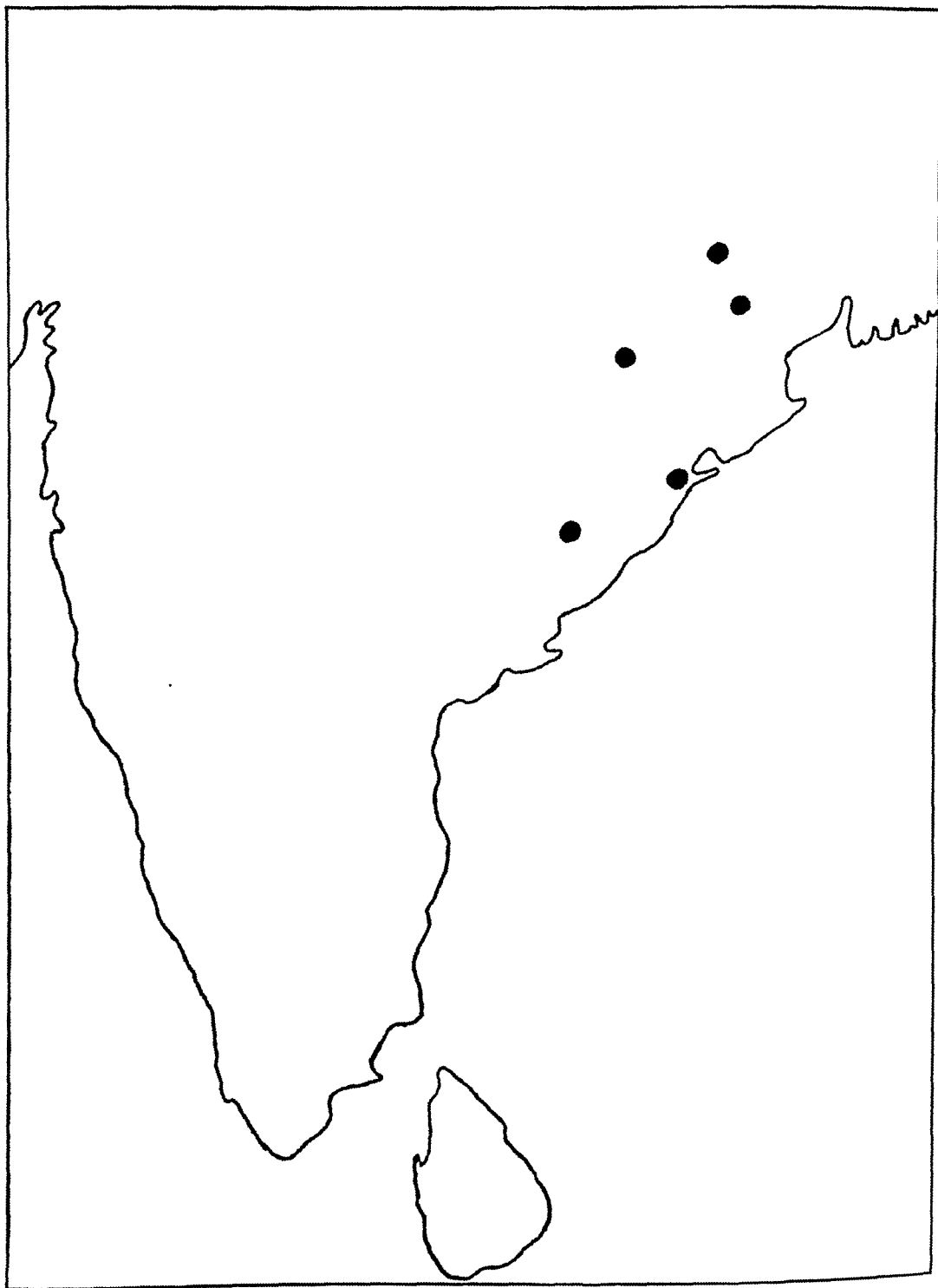
AGE.

The ages of those examined varied from fifteen to forty-five years.

HELMINTHIC INFECTIONS.

Trichuris.—Four, or 18·2 per cent, were 'lightly' infected. The remainder were not infected.

Ascaris.—Eight, or 36·4 per cent, were infected. Of these four were 'light', one was 'moderate', and three were 'heavy' infections.

Hookworm.—Fourteen, or 63·6 per cent, were found to be infected, graded and compared with the eighty garden coolies described in Part II as follows :—

Infection.	Recruits, per cent.	Garden coolies, per cent.
Heavy ..	1 or 4·5	18·7
Moderate ..	5 or 22·7	10·0
Light ..	8 or 36·4	41·3
Nil ..	8 or 36·4	30·0

In the present series the proportion of heavy infections was lower but that of light infections was nearly identical.

HÆMATOLOGICAL DATA.

Hæmoglobin.—In males the hæmoglobin content varied from 10·8 g. to 14·0 g. per 100 c.c. of blood, the mean being 11·77 g.

4 cases had values between 10·5 and 11·49 grammes.
5 ,, ,, 11·5 ,, 12·49 ,,
1 ,, ,, 12·5 ,, 13·49 ,,
1 ,, ,, 13·5 ,, 14·49 ,,

The values in females varied from 9·8 g. to 11·8 g., the mean being 10·85 g.

3 cases had values between 9·5 and 10·49 grammes.
6 ,, ,, 10·5 ,, 11·49 ,,
2 ,, ,, 11·5 ,, 12·49 ,,

The mean for the whole series was 11·31 g. which may be compared with the figure of 10·25 g. which was the combined mean for males and females in the third and fourth decades of life in the coolie series reported in Part II. All these means are markedly lower than any of the numerous observations for both hot and cold climates tabulated by Kennedy (1939). In Table VI have been summarized the observations of Napier (1937), Napier and Bilimoria (*loc. cit.*), and Napier and Majumdar (*loc. cit.*) on coolies in Assam for comparison with my figures.

Compared with their figures, the mean for males in the present group is a little lower than their lowest mean for males, while the female mean is only a little lower than their highest female mean. In other words, the difference between the sexes in this series is not so marked as it is in those quoted.

TABLE VI.

Relevant means of observations on tea-estate coolies published by Napier and his assistants.

Observation.	Napier (1937).	Napier and Bilimoria (1937).	Napier and Majumdar (1938).
MALES.			
Erythrocytes in millions per c.mm.	5·35 / 5·27	..	5·057
Hæmoglobin in grammes per 100 c.c.	11·83 / 12·63	..	12 60
M. C. H. in micro-micrograms.	23·93	..	25·14
M. C. V. in cubic microns	71·29	..	84·93
M. C. H. C. in per cent ..	32·50	..	29·72
FEMALES.			
Erythrocytes ..	4·55 / 4·93	4·55	4·454
Hæmoglobin ..	10·03 / 11·30	10·80	10·40
M. C. H.	23·35	24·50	23·42
M. C. V.	72·30	77·30	82·49
M. C. H. C.	33·07	31·20	28·67

Red blood cells.—In males the red cell counts ranged from 3·85 to 6·26 millions per c.mm , the mean being 5·354.

The female counts ranged from 4·25 to 6·20 millions per c.mm., the mean being 5·226.

The mean for the whole series was 5·290 millions. The mean for males is extremely close to the weighted mean, 5·30 millions, for males in both hot and temperate climates as determined by Kennedy (*loc. cit.*). Compared with the findings of the Napier group, the mean for males is equal to the highest of their determinations and that for females is higher than any of their female means.

White blood cells.—In males the leucocyte counts varied from 4,600 to 11,200 per c.mm., the mean being 8,000.

The range of counts in females was from 6,600 to 12,600, the mean being 8,836.

The mean for the whole series was 8,418 per c.mm. All three means are somewhat higher than those usually quoted in textbooks as the ' normal ' counts and are, also, higher than the means determined for coolies in Labac by Napier and Majumdar (*loc. cit.*), 7,111 for men and 6,456 for women, but all the counts in this series, with one possible exception, were within normal limits.

Mean corpuscular hæmoglobin.—In males values of M. C. H. varied from 17·79$\gamma\gamma$ to 30·65$\gamma\gamma$, the mean being 22·412$\gamma\gamma$.

Values in females ranged from 16·45$\gamma\gamma$ to 25·88$\gamma\gamma$, the mean being 21·102$\gamma\gamma$. The mean for the whole series was 21·757$\gamma\gamma$. The means in the present series are lower than any of the means of the Napier group, but they fall within the possible limits of sampling error.

Mean corpuscular volume.—In males the range of values of M. C. V. was from 65·68 to 113·25 cubic microns, the mean being 79·262.

Values in females varied from 62·41 to 94·39 cubic microns, the mean being 74·860.

The mean for the whole series was 77·061 cubic microns. The figures for M. C. V. determinations by the Napier group are probably not all quite correct since Napier and Majumdar (*loc. cit.*) state that, owing to a defective centrifuge, the means in the 1938 series are too high by about five per cent. If this correction is taken into account the means in the present series agree very well with their observations.

Mean corpuscular hæmoglobin concentration.—In males the values of M. C. H. C. ranged from 25·64 to 29·87 per cent, the mean being 28·293.

The range of values in females was from 25·64 to 30·76 per cent, the mean being 28·169 per cent.

The mean for the series as a whole was 28·231 per cent. Due to the rather lower values of hæmoglobin found in this series, the means for M. C. H. C. values are rather lower than the corresponding findings of the Napier group. Even so, it is clear that the red cells of these recruits are, virtually, fully saturated with hæmoglobin.

CONCLUSIONS.

The results of the investigations made on these recruits agree sufficiently well with the determinations made by Napier and his assistants on tea-estate coolies' in both valleys of Assam to support the suggestion that the partially anæmic state of the 'normal' coolie is shared by the other inhabitants of the villages in rural India whence he comes. There may, or may not, be a further deterioration following residence in Assam, due to various factors peculiar to the Province, but such deterioration, if it occurs, is in no way comparable with the serious alteration which is found in the coolie who has developed clinical 'anæmia'.

As compared with Western standards the rural Indian may be said to have a normal number of red and white cells in the blood, but the hæmoglobin content of the blood is deficient by about twenty-five per cent. Owing to the fact that the size of the red cells is also reduced, saturation of the cells is almost complete. This is the condition said by hæmatologists to result from some interference with the hæmopoietic system in the normoblast-erythrocyte stage.

I have suggested that the severe anæmia of tea-estate coolies is merely an exacerbation of their partially anæmic state and that the ætiology of the two conditions is the same. If this suggestion is correct then the ætiological factor responsible is not peculiar to Assam but must be one which is common both to Assam and to all the various labour recruiting areas from which the tea-estate population is drawn.

SUMMARY.

1. An investigation into the condition of the blood of twenty-two newly recruited tea-estate coolies is described.

2. Steps were taken to ensure that all subjects had not previously resided in Assam, were free from disease, had not come from malarious villages and had not left their homes more than fourteen days previously.

3. The blood standards of rural India can be provisionally stated to be as follows :—

	Male.	Female.
R. B. C. in millions per c.mm. ..	5·354	5·226
Hæmoglobin in g. per 100 c.c. ..	11·77	10·85
W. B. C. per c.mm.	8,000	8,836
M. C. H. in micro-micrograms ..	22·412	21·102
M. C. V. in cubic microns ..	79·262	74·860
M. C. H. C. in per cent	28·293	28·169

PROTOCOL.

Serial number.	Age in years.	Sex.	District of origin.	STOOL.			R. B. C. in millions per c.mm.	Hæmoglobin in g. per 100 c.c.	M. C. H. in micro-micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	W. B. C. per c.mm.	Nucleated red cells.
				Hookworm.	Ascaris.	Trichuris.							
1	28	M.	Koraput ..	M	..	..	6·10	12·7	20·82	69·69	29·87	8,200	..
2	22	F.	,, ..	L	..	..	5·09	11·4	22·39	72·81	30·76	10,000	..
3	25	M.	Ganjam ..	..	..	..	4·67	11·0	23·98	79·35	29·68	4,600	..
4	20	F.	,, ..	..	..	..	6·20	10·2	16·45	62·41	26·36	9,800	..
5	45	M.	Sambalpur	L	..	..	6·26	14·0	22·36	76·61	29·19	6,800	..
6	40	F.	,, ..	L	..	..	4·25	11·0	25·88	84·63	30·58	7,400	..
7	27	M.	Koraput ..	..	L	..	4·85	11·6	23·92	85·40	28·00	7,200	..
8	25	F.	,, ..	L	H	..	6·17	11·8	19·11	66·25	28·87	6,600	..
9	30	M.	,, ..	..	..	..	5·32	11·6	21·81	74·78	29·16	9,200	..
10	27	F.	,, ..	H	M	..	4·33	11·0	25·40	94·39	26·91	7,000	..
11	20	M.	Chaibassa	L	..	..	3·85	11·8	30·65	113·25	27·06	9,200	..
12	18	F.	,, ..	..	L	..	4·66	9·8	21·03	80·69	26·06	12,600	..
13	25	M.	Ranchi ..	M	..	L	6·07	10·8	17·79	70·03	25·64	6,400	..
14	21	F.	,, ..	L	..	..	5·17	11·6	22·44	79·06	28·38	11,000	..
15	30	M.	Ganjam ..	..	..	..	6·14	11·6	18·89	65·68	28·76	11,200	..
16	20	F.	,, ..	L	..	..	5·62	10·8	19·22	70·79	27·15	8,200	..
17	16	M.	Koraput ..	..	..	..	5·11	11·2	21·92	76·79	28·54	10,000	..
18	15	F.	,, ..	..	..	..	5·66	10·2	18·02	70·29	25·64	7,800	..
19	25	M.	,, ..	M	L	L	5·78	12·0	20·76	76·37	27·18	8,200	..
20	22	F.	,, ..	L	L	..	5·58	10·6	18·99	65·43	29·03	8,600	..
21	32	M.	,, ..	M	H	L	4·74	11·2	23·63	83·93	28·14	7,000	..
22	23	F.	,, ..	M	H	L	4·76	11·0	23·19	76·71	30·12	8,200	..

M = Moderate infection.   L = Light infection.   H = Heavy infection.

Part IV.

THE INCIDENCE OF ANÆMIA IN THREE TEA ESTATES.

INTRODUCTION.

In Part II of this paper it has been shown that the most probable factor responsible for the anæmias of tea-estate coolies in Assam is defective nutrition. This Part describes a statistical investigation into the ætiology of these anæmias in the form of a consideration of the relationship between the incidence of anæmia on different estates and certain possible ætiological factors. Three estates were chosen for this study for several reasons : first, they are of comparable size, having working populations of 1,273, 1,070, and 1,494 : secondly, they are each regularly 'combed' every month by the Assistant Medical Officers, all three of whom are reliable men : thirdly, spleen rates had previously been recorded on all three by me personally. I am convinced that, on these estates, all cases of anæmia are detected because, at my periodical musters of the population, I have not been able to detect any anæmics who have not already been listed for treatment. I feel, therefore, that the figures embodied in Table VII are as nearly accurate as is possible in an investigation of this nature. I have not compared hookworm infection rates for the three estates as I have not done stool surveys on two of them and I have found that the infection rates on other estates do not differ very markedly. It is probable that these estates do not differ greatly in this respect, some four-fifths of the working population being infected with hookworm.

TABLE VII.

		ESTATES.		
		'A'.	'B'.	'C'.
Mean total population	{ October 1937 to September 1938.	2,202	1,860	2,337
Mean working population	{ October 1937 to September 1938.	1,273	1,070	1,494
Percentage spleen rates ..	February 1938 ..	49	38	38
Cases of malaria per thousand of total population in three worst months of 1938.	August	47·1	75·0	86·4
	September	34·4	76·1	93·7
	October	41·7	66·1	96·3

TABLE VII—*concld.*

			Estates.		
			'A'.	'B'.	'C'.
Cases of anæmia in working adults per thousand working adults in four quarters.	October to December	..	11·6	29·8	38·7
	January to March	..	14·5	4·9	58·3
	April to June	..	20·3	18·1	62·0
	July to September	..	20·7	12·4	53·2
Cases as above for the twelve months	.. ..		66·8	65·0	215·4
Cases of anæmia in working children per thousand working children in four quarters.	October to December	..	4·9	44·2	54·4
	January to March	..	10·1	11·3	17·5
	April to June	..	12·0	41·0	34·3
	July to September	..	12·9	14·9	20·3
Cases as above for the twelve months	.. ..		39·3	111·9	124·2
Average earnings per hour in annas in four months.	*Adults.* January	..	0·95	0·80	0·45
	April	..	0·85	0·75	0·65
	July	..	1·15	0·70	0·65
	October	..	1·40	0·85	0·90
	Children. January	..	0·95	0·50	0·30
	April	..	0·90	0·45	0·40
	July	..	1·20	0·45	0·50
	October	..	1·60	0·70	0·65
Average earnings per day in annas for two months.	*Adults.* March	..	4·45	4·60	5·10
	September	..	6·90	6·65	8·00
	Children. March	..	3·10	2·70	3·70
	September	..	4·70	6·75	5·00

Malaria has, in the past, been commonly blamed for these anæmias by medical and lay authorities alike and has recently been incriminated by Fairley *et al.* (1938) as the precipitating cause of macrocytic anæmia in Macedonia. In the discussion on this paper Fraser stated that, in his experience, there was a definite relationship between the prevalence of macrocytic anæmia in pregnancy and the prevalence of malaria in tea estates in Assam. The incidence of macrocytic anæmia in pregnancy is not necessarily proportional to that of anæmia in general but for comparison I have tabulated the incidence of clinical malaria during the three worst months of 1938 for the three estates together with their spleen rates. These rates were determined in February 1938 and were obtained by examination of every child between the ages of two and ten years.

The nutritional theory of origin has received more support recently, but is very difficult to test for the average coolie is so unreliable in his statements and so amenable to suggestion and the leading question that accurate diet histories are exceedingly difficult to obtain. I have therefore relied on money earnings as an index of nutritional level. I feel I am justified in doing so for several reasons. A similar proportion of the inhabitants of all three estates rent and cultivate paddy land : the vast majority on all three drink only moderately of country liquor : the proportion of working population to total population on all three is very nearly identical : and, lastly, none of the coolies save much money. The probability is that on all three estates the *proportion* of the total earnings spent on food is nearly identical though the *amounts* may vary quite a good deal. The circumstances regulating the consumption of food may also have a bearing on our problem. If the labour force are compelled to work long hours they are likely to have to consume food which has been hurriedly prepared and, possibly, unsuitable types of food. Moreover, ease of digestion is interfered with and so some loss of absorption may be expected. The daily earnings being known, the amount of money earned per hour becomes an index of the length of the working day. I have therefore tabulated both these items.

Conclusions to be drawn from Table VII.

A glance at Table VII shows that :—

There does not appear to be any constant seasonal variation in the incidence of anæmia.

The anæmia incidence rates in working adults on estates A and B are practically equal and are less than one-third of the rate on estate C.

The *daily* earnings of working adults on estate C are actually higher than those on the others.

The *hourly* earnings of working adults on estate C are lower than those of the others, the difference being especially marked in January.

The anæmia incidence rates in working children on estates B and C are nearly equal and are three times the rate on estate A.

The *daily* earnings of children on estate A are the lowest in the second half of the year and are not the highest in the first half.

The *hourly* earnings of the children on estate A are more than double those on the other two.

Though estate A shows a markedly lower attack rate of clinical malaria than the others, its spleen rate is markedly greater and blackwater fever has occurred from time to time on that particular estate.

DISCUSSION.

The evidence with regard to malaria appears to be inconclusive and there is no positive correlation between the incidence of anæmia and minimal daily earnings. There is, however, a clearly marked relationship between minimal hourly earnings and anæmia incidence : in other words the chances of anæmia increase with the length of the working day.

Unfortunately this fact is capable of more than one interpretation : we may postulate a nutritional anæmia due to inability to consume food at leisure : we may equally well incriminate physical fatigue as the cause of the anæmia : on the other hand we may argue that the anæmic coolie is less efficient and takes longer to fill his allotted task. I do not subscribe to the third suggestion in view of a statement by the Assistant Medical Officer of estate B which, unfortunately, cannot be supported by statistical evidence as the figures are not now available. He says that previous to 1932 the incidence of anæmia on this estate was more than double what it is to-day although a much higher rate of daily wages was paid at that time, long hours being worked. When the financial slump occurred, both hours of work and rates of pay were drastically reduced and, unexpectedly, the incidence of anæmia fell rapidly although no other likely ætiological factors were affected. There has never been any attempt at malaria control on this estate nor has any system of conservancy been adopted nor mass anthelmintic treatment given.

It would appear that, beyond a certain minimum, daily earnings need not be seriously considered in the study of the ætiology of anæmia, a finding which is supported by the fact, noted in Part II, that variations in the coolie standard of living, so long as they were within the normal range, did not affect the hæmoglobin level. The important factor appears to be the length of the working day but there is no indication as to whether fatigue is the vital feature or the inability of the coolie to consume and digest suitably chosen and adequately cooked food.

Part V.

DATA COLLECTED FROM TWENTY CASES OF MARKED ANÆMIA IN NON-PREGNANT WOMEN.

INTRODUCTION.

Since, to the best of my knowledge, the only series of cases of anæmia, not complicating pregnancy, in tea-estate coolies, reported in the literature is that of Napier and Das Gupta (1937a) it seemed advisable to confirm their findings in this district, which lies about one hundred miles away from the scene of their investigations.

For this study twenty women who had been admitted to hospital on one estate for anti-anæmic treatment were examined. They had been selected as anæmic by the Assistant Medical Officer of the estate during his monthly inspections of the labour force and, as opportunity offered for carrying out a hæmatological examination, such patients as were newly admitted to hospital and had received no treatment were subjected to examination. In this way no conscious selection of cases occurred and the findings reported in this paper are probably representative of the anæmia of non-pregnant women as seen in this district as a whole. As in Napier and Das Gupta's series the selection by the Assistant Medical Officer was made on clinical grounds and confirmed by a reading on the Tallqvist scale, a hæmoglobin value of fifty per cent or lower being regarded as indicating a severe degree of anæmia. The usual reasons for selection were general malaise (the woman being regarded as lazy), dyspnœa on exertion, and, occasionally, some puffiness of the face. In all cases there was marked pallor of the mucous membranes. Night-blindness was common but other signs of gross vitamin deficiency were absent.

Each case was submitted to a fairly complete physical examination and to stool and hæmatological examinations, following the technique described in Part I. All examinations were carried out between the hours of two and four in the afternoon.

As a result of the hæmatological investigations, the cases divided themselves into two well-defined groups. The larger group of eighteen cases contained no woman with mean corpuscular volume greater than 85 cubic microns : the highest actual value was 81·52. This I have designated the microcytic-hypochromic group. The smaller group had higher values of M. C. V. and will be discussed as the macro-microcytic group.

FINDINGS IN THE MICROCYTIC-HYPOCHROMIC GROUP.

Age.—The age distribution of the cases was as follows :—

7 cases were between 15 and 19 years.
2 ,, ,, 20 ,, 24 ,,
3 ,, ,, 25 ,, 29 ,,
4 ,, ,, 30 ,, 34 ,,
2 ,, ,, 35 ,, 40 ,,

The preponderance of cases in the earliest age group is interesting as it confirms a finding by Napier and Das Gupta (1937a) in their ' first series '.

Residence in Assam.—None of the women were indigenous Assamese though two were born in Assam. Of the remainder :—

1 had resided in Assam less than 1 year.
5   ,,   ,,   ,,   5 years.
10   ,,   ,,   more than 5   ,,

This distribution is reasonably in accordance with the general distribution of the population of this particular estate.

Previous anæmia.—Of the eighteen women no less than fifteen had been previously treated for anæmia. On this estate the treatment of anæmia is particularly thorough : all cases are given adequate anthelmintic treatment as in-patients and are also treated intensively with iron and a full diet. Evidently these recurrent cases relapse after returning to their own homes : I am not, at the moment, prepared to offer any explanation of this fact.

Condition of spleen and liver.—In no case was the liver enlarged but the spleen was palpable in two women, both of whom had been previously treated for anæmia.

History of recent malaria.—In only two cases was there a history of an attack of ' fever ' during the preceding three months which, in every instance, covered at least two months of the most active period of transmission. Both these cases were in the previously treated group and one had an enlarged spleen. As the spleen rate *in children* on this estate is 38 per cent, the presence of two instances of splenomegaly and two histories of recent malaria is probably merely what would be observed in any unselected sample of the general adult population.

Hookworm infection.—This series showed a 100 per cent infection rate distributed as follows :—

5 cases had light   infections.
2   ,,   moderate   ,,
11   ,,   heavy   ,,

The proportion of heavy infections is much higher than would be expected in a group of healthy persons (*see* Part II of this paper) but is in accordance with the experience of Napier and Das Gupta (1937a).

Ascaris and trichuris infections.—Light infections of roundworm were found in two women and one had a moderate infection of whipworm.

Urinary abnormalities.—No trace of either sugar or albumin was found in the urine of any case.

Lungs.—Clinical examination of the lungs failed to disclose any abnormality in any case.

Cardiac enlargement.—No case showed any gross enlargement of the heart.

Cardiac sounds.—A hæmic bruit was detected in eleven cases. Otherwise no abnormalities were found and examination of the cardiovascular system failed to reveal any evidence of syphilis.

Pulse rate.—The pulse rate, taken at rest, and after steps had been taken to allay nervous excitement, was above 80 per minute in thirteen cases.

The tendency to a slightly increased pulse rate is presumably due to some degree of oxygen lack.

Œdema of the extremities.—Obvious œdema of the feet and legs was not observed in any case.

Red cell counts.—The number of erythrocytes per c.mm. of blood varied from 2·04 to 5·01 millions, the mean being 3·41 millions. The distribution was fairly central.

Reference to the figures quoted in Part III of this paper will show that there was in the majority of these cases a gross deficiency in the number of red cells in the peripheral blood.

Hæmoglobin content.—The lowest value of hæmoglobin was 2·6 g. per 100 c.c. of blood and the highest was 9·0 g., the mean being 5·08 g. The distribution was as follows :—

Hæmoglobin in 1 case was between 2·0 and 2·9 grammes.
,,   ,, 5   ,,   ,,   3·0 ,, 3·9   ,,
,,   ,, 4   ,,   ,,   4·0 ,, 4·9   ,,
,,   ,, 3   ,,   ,:   5·0 ,, 5·9   ,,
,,   ,, 1   ,,   ,,   6·0 ,, 6·9   ,,
,,   ,, 2   ,,   ,,   7·0 ,, 7·9   ,,
,,   ,, 2   ,,   ,,   8·0 ,, 9·0   ,,

Reference, again, to Part III will show that in all cases there was a deficiency of hæmoglobin which, in the majority, amounted to a gross defect.

Mean corpuscular hæmoglobin.—The values of M. C. H. in micro-micrograms ranged from 9·86 to 19·94, the mean being 14·67 and the distribution was central.

All the cases can be said to be hypochromic by any standard.

Mean corpuscular volume.—The lowest value of M. C. V. in cubic microns was 59·86 and the highest was 81·52, the mean being 73·58.

The mean value of M. C. V. was only slightly below that for female new coolies (*see* Part III) but all cases had values below 85 cubic microns. Though Napier and Majumdar (*loc. cit.*) have suggested that any case with a value of M. C. V. over 75 cubic microns should be regarded as normocytic, I cannot subscribe to such a low limit and consider that, compared with the normal values determined for

Britain, America, and urban India, a M. C. V. even as high as 81·52 cubic microns can safely be regarded as indicating a microcytic condition. If we were considering cell size in relation to that determined for the normal healthy tea-estate coolie we would have to fix a much lower level than 75 cubic microns above which any case would be classed as normocytic. I prefer to regard the general coolie population as having a microcytic condition of the red cells and this particular series of cases as being all instances of microcytic-hypochromic anæmia.

Mean corpuscular hæmoglobin concentration.—The lowest value calculated was 15·11 per cent, the highest 26·21 per cent, and the mean 19·89 per cent.

In all eighteen cases the saturation of the red cells with hæmoglobin was deficient.

White cell counts.—The number of leucocytes per c.mm. ranged from 2,800 to 16,000, the mean being 6,090. In thirteen cases the number was between 4,000 and 8,000, so that it may be said that the counts did not materially differ from those usually considered to be 'normal'.

Stained films.—All the films showed fairly well-marked anisocytosis with a preponderance of small cells, large numbers of the red cells staining badly and vacuolation being marked. In two cases occasional normoblasts were seen but megaloblasts were never seen in this series.

DISCUSSION.

All the cases in this group are hypochromic by any standard and are microcytic by all ordinary standards though the average value of M. C. V. is only slightly below that found in healthy coolies. Both red cells and hæmoglobin are in defect but the defect is more marked in the case of the latter so that very low values of M. C. H. C. are the rule. This group of cases can, therefore, be classified as examples of microcytic-hypochromic anæmia.

On the findings as stated, there is no reliable epidemiological evidence that this anæmia is due to malaria, acute or chronic, but the position with regard to hookworm infection is not so clear. While a high proportion of heavy infections was found in this group the fact remains that it is very difficult to find any correlation between hæmoglobin level and the degree of infection as judged by the number of ova in the stool. If hookworm infection were the primary cause of this type of anæmia the only possible explanation of the enormous liability to recurrence would be an assumption that these persons are especially susceptible either to hookworm disease or to hookworm infection. The first of these assumptions implies that some toxæmia results from infection by hookworm, an hypothesis for which there is very little evidence. The frequency of hookworm infection in this class of the population suggests that it is unlikely that any particular persons are especially liable to infection : it is much more likely that all are equally susceptible.

It would seem to be more likely that some as yet unknown cause renders certain people predisposed to anæmia and that, in these persons, the exciting cause may be a heavy infection with hookworm.

FINDINGS IN THE MACRO-MICROCYTIC GROUP.

The two cases in this group were ten and thirty-five years of age : one had lived three years in Assam, the other seven. Both had been previously anæmic but neither gave a history of recent malaria and in neither case was the spleen enlarged. Neither had any cardiac enlargement but in both cases the pulse was fast and hæmic murmurs were audible. Œdema of the feet was noted in one case. The lungs of both appeared to be normal and no abnormalities were found in the urine. Both were heavily infected with hookworm.

In both cases the red cell count was only a fraction over two millions and hæmoglobin values were very low giving low values of M. C. H. and M. C. H. C. The values of M. C. V. were 91·27 and 107·46 cubic microns. White cell counts were within normal limits. The stained films in both cases were notable for the extreme degree of anisocytosis seen : vacuolation of the cells was marked : no normoblasts or megaloblasts were observed.

DISCUSSION.

These two cases obviously present broadly similar characteristics though on a consideration of M. C. V. findings alone one could be. classed as normocytic and the other as macrocytic. Moreover, these two cases present certain features common to the microcytic group already described : both are hypochromic with defective saturation of the red cells and both were heavily infected with hookworm but did not appear to be suffering from chronic malaria. Neither of them present the picture of macrocytic anæmia as described by Wills (1932) or of macrocytic-hæmolytic anæmia described by Napier (1936) and recently investigated by Fairley *et al.* (*loc. cit.*). On the other hand, both clinically and hæmatologically, these two women were among the most severely affected of the twenty cases described and one of them was the only case with obvious œdema of the feet. I consider that these two cases are examples of a definite clinical entity which I propose to term micromacrocytic anæmia and which I believe to be identical with one type of anæmia seen in pregnant women. In a later part of this paper I shall advance arguments in support of this attitude.

There is a consensus of opinion that the fundamental cause of macrocytic anæmia in the tropics is a deficiency of the vitamin-B complex in the diet. There are also good reasons for thinking that some dietary defect is the fundamental cause of microcytic anæmia also. It is possible that the diet of certain individuals is so grossly defective that both dietetic factors are in deficient amounts, thereby producing an anæmia with some of the characteristics of both main types.

PROTOCOL.

Serial number.	Age in years.	Years in Assam.	Previously anaemic?	Recent malaria?	Spleen.	Œdema present?	Haemic bruit?	Stool. Hookworm.	Stool. Ascaris.	Stool. Trichuris.	R. B. C. in millions per c.mm.	Haemoglobin in g. per 100 c.c.	Cell volume per cent.	W. B. C. in thousands per c.mm.	M. C. H. in micro-micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	Remarks on film.
1	16	16	Y				Y	L		M	3·28	4·8	24·52	16·0	14·63	74·77	19·57	N
2	16	16					Y	H			3·45	4·6	23·43	7·4	13·33	63·93	19·63	
3	25	6	Y	Y	P		Y	L			3·22	5·2	24·52	2·8	16·15	76·16	21·20	
4	22	22	Y				Y	M			2·90	5·0	23·43	3·2	17·24	80·81	21·33	
5	20	6	Y				Y	H			3·51	5·6	28·43	3·4	15·95	80·74	19·76	
6	28	2	Y					L			2·04	2·6	15·81	4·2	12·74	77·50	16·45	
7	35	22	Y				Y	H			4·17	8·0	33·79	4·2	19·18	81·03	23·67	N
8	16	2						H			3·75	6·6	28·34	5·4	17·60	75·57	23·29	
9	35	6	Y		P		Y	L	L		4·46	7·0	26·70	5·4	15·69	59·86	26·21	
10	34	24	Y				Y	H	L		3·38	3·6	23·98	5·8	10·65	70·94	15·84	
11	16	6	Y				Y	H			3·81	4·2	27·79	6·4	11·02	72·95	15·11	
12	16	2	Y				Y	H			2·61	3·0	18·53	6·4	11·48	70·99	16·19	
13	30	¾					Y	L			3·65	3·6	22·89	6·6	9·86	62·71	15·73	
14	35	15		Y				M			5·01	9·0	35·42	6·8	17·96	70·71	25·40	
15	16	18	Y					H			2·69	3·6	20·16	7·4	13·38	74·96	17·85	
16	32	20	Y				Y	H			3·61	7·2	29·43	7·8	19·94	81·52	24·46	
17	30	2	Y					L			2·44	3·6	19·07		14·75	78·15	18·88	
18								H			3·37	4·2	23·98	10·2	12·50	71·15	17·52	

Micro-macrocytic group.

Serial number.	Age in years.	Years in Assam.	Previously anaemic?	Recent malaria?	Spleen.	Œdema present?	Haemic bruit?	Stool. Hookworm.	Stool. Ascaris.	Stool. Trichuris.	R. B. C. in millions per c.mm.	Haemoglobin in g. per 100 c.c.	Cell volume per cent.	W. B. C. in thousands per c.mm.	M. C. H. in micro-micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	Remarks on film.
19	35	3	Y			Y	Y	H	M		2·09	2·9	19·07	6·4	13·87	91·27	15·20	A
20	10	7	Y				Y	H			2·13	4·4	22·89	4·6	20·66	107·46	19·22	A

L = Light infection.
N = Normoblasts present.
A = Marked anisocytosis.

Y = Yes.
P = Palpable.
H = Heavy infection.
M = Moderate infection.

Part VI.

THE INCIDENCE OF ANÆMIA IN THE LAST QUARTER OF PREGNANCY IN TEA-ESTATE COOLIES.

Introduction.

The problem of the ætiology of the anæmias complicating pregnancy in India is still far from solution and our knowledge of many of the elementary facts regarding their incidence is incomplete. Estimates as to the incidence of anæmia, based on hospital admissions, have been made by Mitra (1937), Choudhury and Mangalik (1938), and Chatterjee (1938). All these estimates are liable to the usual objections raised against statistics based on a selected population rather than on a total population. The very fact that a group of women, who are members of a community who are normally confined in their own homes, choose to be delivered in hospital vitiates their being considered to be a true sample of the population from which they were drawn. Napier and Das Gupta (1937b) made an estimate of the incidence of anæmia complicating pregnancy in a controlled population (the inhabitants of a group of tea gardens in Assam) but it would appear from their account that in each individual only one estimation of hæmoglobin was made and that the investigation only continued for a short period. My position as medical officer to a group of tea estates in Upper Assam has rendered it possible for me to study this and other problems connected with the ætiology of anæmia in pregnant women in a population under a fair measure of control.

The group of fourteen estates in my medical charge comprises a total population of the coolie class of, approximately, fifteen thousand souls. During the year 1938 the number of deliveries occurring after the twenty-eighth week of pregnancy was 758. All these estates grant varying periods of optional leave to pregnant and parturient women but, though all avail themselves of the privilege after delivery, some women prefer to carry on working until labour commences. As a result, eighty-three (or approximately eleven per cent) did not take ante-partum leave and so were not seen by me before delivery. The remaining 675 women were all seen by me on at least one occasion before delivery and, in fact, the average number of interviews per case was about six. At each interview a hæmoglobin estimation was made by the Tallqvist method, all estimations being made by myself in order to avoid the inevitable fallacies associated with two or more persons using a technique involving the matching of colours. Every woman who, at any time between the thirtieth week of pregnancy and full term, had a Tallqvist reading of fifty per cent or lower was regarded as being anæmic for the purpose of this inquiry. I would emphasize that a very large number of these women showed a low reading only at some of their interviews and that, accordingly, had a single estimation only been made, many cases of anæmia would have been missed. I would also emphasize that in this inquiry

only women in the last quarter of pregnancy were dealt with, so no attempt has been made to estimate the period of onset of anæmia. A more exact inquiry on this point will be made the subject of a later paper.

INCIDENCE OF ANÆMIA.

Of the 675 women seen, 184 had one or more readings of fifty per cent or lower on the Tallqvist scale. Even if we assume that the eighty-three women who were not interviewed were all healthy, the percentage incidence of anæmia was 24·27. This is a much higher figure than any previous estimate for the reasons I have mentioned and is probably at least double the incidence of anæmia in the non-pregnant population. For example, the combined incidence of anæmia in working adults on the three estates considered in Part IV of this paper was 12·75 per cent. In the same three estates the combined incidence of anæmia in pregnancy was 30·10 per cent.

The incidence varied enormously in different estates—the lowest incidence being 6·38 per cent and the highest 48·21 per cent.

3 estates had an incidence below 10·0 per cent.
2 ,, ,, ,, over 10 ,, but below 20 per cent.
5 ,, ,, ,, ,, 20 ,, ,, 30 ,,
2 ,, ,, ,, ,, 30 ,, ,, 40 ,,
2 ,, ,, ,, ,, 40 ,,

This variation is difficult to explain. No exact correlation between anæmia incidence in pregnant and non-pregnant women could be discovered nor was there any correlation between spleen rates and the incidence of anæmia in pregnancy. An exact correlation between hours of work and anæmia incidence, such as was demonstrated in Part IV, could not be established, but I believe that a sociological survey on those lines would reveal some interesting facts.

SEASONAL INCIDENCE OF ANÆMIA.

The question whether the incidence of anæmia is variable from season to season has, again, not been satisfactorily answered. Mitra (*loc. cit.*) and Chatterjee (*loc. cit.*) both considered that, in Bengal, the incidence was higher during the latter half of the year, whereas Choudhury and Mangalik (*loc. cit.*), also working on hospital admissions, were unable to find any seasonal variation.

I have set out in Table VIII the total number of deliveries occurring in each quarter of 1938 in the estates in my medical charge together with the number of women delivering in each quarter who, at any time during the period between the 32nd week of pregnancy and delivery, had shown a Tallqvist scale reading of fifty per cent or lower.

TABLE VIII.

Total deliveries and incidence of anæmia in four quarters of 1938.

Quarters.	Total deliveries.	Anæmia cases.	Incidence, per cent.
January to March ..	194	41	21·13
April to June ..	125	29	23·20
July to September ..	209	59	28·24
October to December ..	231	55	23·81

It will be noted that the number of anæmic women delivering in the second half of the year is very much greater than the number in the first half but that this is largely due to the much larger number of total deliveries in the second half of the year. The third quarter does stand out as having a higher incidence than any other and the difference is, in fact, statistically significant. Our problem of ætiology is, however, still far from solution as this difference can be interpreted in several different ways. The evidence produced by other workers tends to show that it is probable that the majority of these cases of anæmia commence to be anæmic at about the 32nd week. If we accept this we are entitled to assume that a very large number of these women became anæmic in June. The rapid response to treatment of the majority of cases of anæmia in Assam indicates that the immediate cause of the anæmia is only shortly antecedent even if the predisposing cause is of long duration. One may, therefore, assume that in these cases the immediate cause was something operative in May or early June. This might be malaria, since, in populations associated with permanent or semi-permanent streams, that period usually shows a ' peak ' on the malaria-incidence graph. It may be mentioned here that the bulk of the population which is being dealt with is associated with such streams. On the other hand, as mentioned above, I have been unable to find any evidence of increased incidence on grossly malarious estates as compared with others which are more healthy from that point of view. Equally with malaria one may blame unhealthily long working hours since that period is a very busy one on these estates and, in addition, many of the coolies are engaged at that time in the cultivation of their own rice land. This is in the nature of a ' spare-time ' job and still further increases the length of the working day. The violent change in climatic conditions from cool dry winter to hot humid summer cannot be ignored nor can the possibility of the change of season altering the dietetic habits of the population. This uncertainty may, perhaps, be somewhat resolved when further evidence as to the periods of origin of anæmia is available.

INCIDENCE OF ANÆMIA IN DIFFERENT AGE GROUPS.

Unfortunately no record was kept of the ages of the non-anæmics so that only an indirect estimation of the age group incidence could be made. Of the 184 women found anæmic, a history was taken from, and a clinical examination was made of, 158, that is to say, approximately 86 per cent of the total number. Of these :—

25 were under 20 years of age.

90 ,, over 20 but under 30 years of age.

43 ,, ,, 30 years of age.

This finding, that 57 per cent of the cases were in the age group 20–29 years, is in accordance with the experience of other workers and is what one would reasonably expect since that decade is the maximum child-bearing period of the coolie class. It is probable that the actual incidence per cent of deliveries is fairly uniform throughout the child-bearing period.

INCIDENCE OF ANÆMIA IN DIFFERENT PREGNANCIES.

This again, unfortunately, could only be estimated indirectly. Of the 158 women whose histories were taken :—

42 were in their 1st pregnancy.

37 ,, ,, 2nd ,,

23 ,, ,, 3rd ,,

24 ,, ,, 4th ,,

13 ,, ,, 5th ,,

19 ,, ,, 6th or subsequent pregnancy.

Exactly fifty per cent of the total were in either their first or second pregnancy. Now rather more than fifty per cent of women of this class bear three or more babies so that in any unselected group of pregnant women the chances are that less than fifty per cent are in their first or second pregnancies. Therefore we are justified in considering that anæmia is more frequently met with in the first two pregnancies. This finding is in accordance with the views of other workers in this field.

THE EFFECT OF ANÆMIA ON MATERNAL MORTALITY.

Of the 184 women found to be anæmic before they delivered, ten died during, or shortly after, their confinements. This gives a fatality rate of 5·43 per cent but that is not the true fatality rate of the disease, even during the last quarter of pregnancy only, since several women who died undelivered have been excluded from consideration in this inquiry which is based entirely on deliveries. Even so, it is almost double the mortality rate based on total deliveries and is nearly three times the rate in non-anæmics. Table IX illustrates these points and it will be seen that anæmia is by far the most important cause of death associated with childbirth. The total maternal mortality rate is a little higher than the general

death rate for the district, usually about 20 per 1,000, and the maternal mortality among the non-anæmics is approximately equal to the general death rate. This is in accordance with certain clinical impressions which one had already formed, namely, that the incidence of the toxæmias of pregnancy is lower in these people than in Western countries and that their resistance to septic infection is high and, lastly, that the non-anæmics stand the physical strain of labour very well indeed. Removal of anæmia as a cause of death would render childbirth no more hazardous to the tea-estate coolie than her normal existence.

TABLE IX.

Maternal mortality rates for 1938.

Total deliveries :—	758	Anæmic deliveries :—	184	Non-anæmic deliveries :—	574
Deaths	21	Deaths	10	Deaths	11
Rate per thousand ..	27·70	Rate per thousand ..	54·34	Rate per thousand ..	19·16

EFFECT OF ANÆMIA ON THE FŒTUS.

The common experience that maternal anæmia does decrease the chances of fœtal survival is amply confirmed by Table X.

TABLE X.

Premature and stillbirth rates in 1938.

NON-ANÆMIC DELIVERIES, 574.			ANÆMIC DELIVERIES, 184.		
Class.	Number.	Per cent.	Class.	Number.	Per cent.
Live premature births ..	41	7·13	Live premature births ..	21	11·41
Premature stillbirths ..	17	2·93	Premature stillbirths ..	16	8·69
Full-term stillbirths ..	30	5·22	Full-term stillbirths ..	16	8·69
Total premature births ..	58	10·10	Total premature births	37	20·10
Total stillbirths ..	47	8·19	Total stillbirths ..	32	17·38

Both the premature birth rate and the stillbirth rate are doubled in the anæmic group as compared with the non-anæmic group, but the principal increase is in the number of premature stillbirths. The risk of this accident appears to be trebled when the mother becomes anæmic.

SUMMARY.

1.   An inquiry into the incidence of anæmia complicating pregnancy in the coolie population of a group of tea estates over a period of twelve months is described.

2.   The incidence found (24·27 per cent of all deliveries after the 28th week of pregnancy) was higher than that noted by other workers for reasons which are explained.

3.   The incidence varied enormously in different estates and no reason for this variation could be formulated.

4.   The influence of season on incidence was studied and, while the principal effect of the seasons was found to be on the total birth rate, there was also a definite increase in the incidence of anæmia in those women who delivered during the third quarter of the year.

5.   The findings of previous workers as to the risks in various age groups and in various pregnancies were confirmed.

6.   The effect of anæmia on maternal mortality was studied and this disease was found to be easily the most important cause of maternal deaths in this class. With this risk removed, childbirth would cease to be an added risk to life.

7.   The effect of maternal anæmia on the fœtus was studied and the observation made that the principal added risk was that of premature stillbirth.

Part VII.

CLINICAL AND HÆMATOLOGICAL DATA OBTAINED FROM 182 COOLIES FOUND TO BE ANÆMIC DURING THE LAST QUARTER OF PREGNANCY.

INTRODUCTION.

In the course of the investigation reported in Part VI of this paper 184 women were found to be anæmic at some time during the last quarter of pregnancy and of these I was able to obtain histories from, and make a clinical examination of, some 158. In addition I have records of 21 women who were anæmic and more than thirty weeks' pregnant but who had not delivered at the end of December 1938. Records had also been obtained of three women, anæmic and more than thirty weeks' pregnant, who had died undelivered.

In all cases the hæmoglobin was estimated accurately with the Sahli instrument, a history obtained and a clinical examination carried out. In rather more than half the stools were examined and the urine was tested in a similar number of cases. In twenty-six simple blood counts were made and in a further seventy-eight, full hæmatological examinations were carried out. In the great majority stained films of the blood were examined and an attempt made to assess the character of the anæmia. In all examinations the technique described in Part I was followed.

DISCUSSION OF THE CLINICAL FINDINGS.

Of the total number, thirteen women died, a new feature compared with the series of non-pregnant anæmics already described.

Residence in Assam.—Among the non-fatal group, the distribution of degrees of residence was roughly in accordance with that of the population of the district. Among the fatal group, no case had resided in Assam less than one year nor was any case Assam-born: half had resided less than five years and the remainder more than that period.

Previous anæmia.—Whereas 85 per cent of the non-pregnant group gave a history of previous anæmia, only 23·1 per cent of the fatal group and 26·6 per cent of the non-fatal group gave such a history. This finding suggests that anæmia in pregnancy is largely non-recurrent and may be a product of the pregnancy itself.

Recent malaria.—A history of recent ' fever ' was noted in only 10 per cent of the non-pregnant group but 24·2 per cent of the non-fatal group gave such a history and 46·1 per cent of the fatal cases had recently had malaria.

Splenic enlargement.—Taking into account the rarity in this district of kala-azar and the negative formol-gel reactions found, splenic enlargement was taken to be indicative of chronic malaria. Splenic enlargement was found in 10 per cent

of the non-pregnant group, in 26 per cent of the non-fatal group, and in 53·8 per cent of the fatal group.

Hepatic enlargement.—No non-pregnant case was found to have enlargement of the liver and this condition was only found in 3·5 per cent of non-fatal cases but it was noted in 15·4 per cent of fatal cases. In each case of hepatic enlargement the spleen was also enlarged, with one exception and in no case was there any evidence of gross cardiac incompetence or of amœbic infection. It would appear to be probable that the hepatic enlargement was due to malarial hepatitis following long-standing chronic malaria.

We may now consider the implications of these findings regarding malaria. During 1938 approximately ten thousand cases of malaria occurred among the total coolie population of fifteen thousand souls in this district, an *average* of 2,500 per three months. The chances are, then, that in any hundred persons from fifteen to twenty would have given a history of recent malaria : the 46 per cent incidence in the fatal group is, therefore, much higher than the expected incidence even if the incidence in non-fatal cases is not much higher. Splenic enlargement was twice as common in the fatal group as in those who recovered and was twice as common in those pregnant as in those not pregnant. Again, hepatic enlargement was at least four times as common in those who died as in those who recovered. I think it is justifiable to say that malaria probably plays a greater part in the ætiology of anæmia in pregnant women than it does in those not pregnant and that it is an important ætiological factor in the most serious and fatal type of anæmia.

Pulmonary complications.—Only a few cases showed pulmonary complications which were more frequently met with in the fatal group, probably an expression of the severity of the disease.

Œdema of the extremities.—Œdema was noted in 77 per cent of those who died and in 34 per cent of those who recovered, compared with only 5 per cent in those not pregnant. Moreover, the fatality rate in those with œdema was 15 per cent against 2·5 per cent in those without œdema. Œdema was very seldom seen in the pregnant women who were not anæmic, so that it would appear to have some prognostic significance. Half of the ten women with œdema who died had also an enlarged spleen but only two had any cardiac enlargement and none showed signs of gross cardiac defect. It is improbable that malaria is directly responsible for the œdema and the fact that many cases with a high degree of anæmia do not develop œdema suggests that anæmia, also, is not directly responsible. I suggest that the œdema may be a symptom of some other condition, possibly causally connected with the anæmia and that the grave prognosis associated with a combination of splenic enlargement and œdema is due to the fact that more than one pathological entity is present.

Cardiac abnormalities.—Pulse rates in both groups showed the same slight degree of tachycardia as was encountered in the non-pregnant anæmics and hæmic bruits were heard in a similar percentage in all three groups. In both pregnant groups some 15 per cent had some enlargement of the heart, whereas this was never noted in those not pregnant. Evidently no prognostic significance attached.

Urinary abnormalities.—Albumin was found in small quantities in a similar proportion of cases in both fatal and non-fatal groups.

Helminthic infections.—Of the 99 stools examined—

10 showed	*nil*	hookworm infection.
34	,,	heavy	,,	,,
22	,,	moderate	,,	,,
33	,,	light	,,	,,

In addition, 66 per cent were ascaris-free and 72 per cent were trichuris-free.

DISCUSSION OF THE HÆMATOLOGICAL FINDINGS.

On analysing the results obtained from the blood examinations, the cases fell automatically into five groups as follows :—

A. A microcytic-hypochromic group of 36 cases in which the anæmia was similar in type and degree to that usually seen in non-pregnant women, together with a group of 16 markedly hypochromic cases whose blood films showed obvious microcytosis with no nucleated red cells.

B. A normocytic-hypo-orthochromic group of 13 cases with M. C. V. values ranging from 83·05 to 110·38 cubic microns and M. C. H. values varying between 16·55 and 31·82 micro-micrograms. This group was distinguished by a marked absence of anisocytosis in the stained films together with an absence of nucleated reds. On these grounds three further orthochromic cases have been included in this group.

C. A micro-macrocytic-hypo-orthochromic group of 24 cases generally corresponding to the 'smaller group' of non-pregnant anæmics already described. This group was distinguished by marked anisocytosis and a relative frequency of nucleated reds in the stained films with a M. C. H. under 30 micro-micrograms. On these grounds one further case was added to this group.

D. A hyperchromic group of 10 cases which were almost certainly all macrocytic. In five whose blood cell volume was estimated, the M. C. V. values were high and their films like the other hyperchromic cases all showed the same characteristics, namely, marked anisocytosis with many macrocytes but few microcytes together with the presence of normoblasts and megaloblasts. Three further cases, whose films had the same characteristics, were added to this group.

E. A group of 75 women whose hæmatological data were insufficiently complete to allot them to any one of the first four groups.

The hæmatological findings of the first four groups have been given in Tables XI, XII, XIII, and XIV, and it is proposed to discuss each group in greater detail in turn.

TABLE XI.

Range and means of hæmatological observations in the microcytic group.

Estimation.	Highest determination.	Lowest determination.	Mean.
Erythrocytes in millions per c.mm.	5·14	2·14	3·522
Hæmoglobin in g. per 100 c.c. ..	9·0	3·4	5·96
Leucocytes per c.mm. ..	16,000	3,000	7,961
M. C. H. in micro-micrograms ..	23·95	11·04	17·303
M. C. V. in cubic microns ..	84·82	56·52	73·879
M. C. H. C. in per cent ..	28·50	15·99	22·237
Blood films	No nucleated red cells seen: vacuolation marked: many microcytes.		

TABLE XII.

Range and means of hæmatological observations in the normocytic-hypo-orthochromic group.

Estimation.	Highest determination.	Lowest determination.	Mean.
Erythrocytes in millions per c.mm.	3·66	1·32	2·486
Hæmoglobin in g. per 100 c.c. ..	7·8	3·8	5·98
Leucocytes per c.mm. ..	10,600	3,000	6,775

TABLE XII—*concld.*

Estimation.	Highest determination.	Lowest determination.	Mean.
M. C. H. in micro-micrograms ..	31·82	16·55	24·753
M. C. V. in cubic microns ..	110·38	83·05	95·815
M. C. H. C. in per cent ..	35·55	13·52	24·712
Blood films ..	Normoblasts seen in one case : no film showed any degree of anisocytosis.		

TABLE XIII.

Range and means of hæmatological observations in the micro-macrocytic-hypo-orthochromic group.

Estimation.	Highest determination.	Lowest determination.	Mean.
Erythrocytes in millions per c.mm.	3·18	1·46	2·271
Hæmoglobin in g. per 100 c.c. ..	7·5	3·4	5·04
Leucocytes per c.mm. ..	13,400	3,000	7,533
M. C. H. in micro-micrograms ..	29·66	15·59	22·504
M. C. V. in cubic microns ..	147·74	87·29	101·691
M. C. H. C. in per cent ..	29·91	15·49	22·987
Blood films ..	Normoblasts alone seen in three cases : normoblasts and megaloblasts seen in six cases : all films showed excessive anisocytosis.		

TABLE XIV.

Range and means of hæmatological observations in the macrocytic-hyperchromic group.

Estimation.	Highest determination.	Lowest determination.	Mean.
Erythrocytes in millions per c.mm.	1·87	0·73	1·473
Hæmoglobin in g. per 100 c.c. ..	6·9	1·9	5·15
Leucocytes per c.mm. ..	12,600	3,200	6,640
M. C. H. in micro-micrograms ..	47·26	32·62	37·306
M. C. V. in cubic microns ..	164·25	121·11	140·352
M. C. H. C. in per cent ..	32·35	24·46	26·986
Blood films ..	All films showed obvious megalocytosis : all contained both normoblasts and megaloblasts.		

MICROCYTIC-HYPOCHROMIC GROUP.

This group is very similar to the main group of non-pregnant anæmics already described. All are hypochromic and all whose cell volume was estimated are microcytic, taking 85 cubic microns, again, as the upper limit for this class. The distribution and means of red cell counts and of M. C. V. are closely equivalent to those found in the non-pregnant women. Of red cells 66 per cent had between 3·0 and 4·49 millions per c.mm.: 69 per cent of the M. C. V. values lay between 65·0 and 79·9 cubic microns. The means of values of hæmoglobin, M. C. H., and M. C. H. C., are slightly higher in each case than in the non-pregnant group but this is probably because a number of women were included in this series who would not have normally been recognized as anæmic. Hæmoglobin values were distributed fairly evenly between 4·0 and 7·9 grammes but in the case of M. C. H. values 71 per cent were concentrated between 15·0 and 20·99 micrograms and 66 per cent of M. C. H. C. determinations were between 19·0 and 24·99 per cent. The white cell counts show no great departure from the probable findings in any series of 'normal' people.

The proportion of histories of previous anæmia and of recent malaria and the incidence of splenic enlargement were rather lower in this group than in the whole

series but the incidence of hookworm infections and heavy infections in particular was almost identical. There were two deaths in this group, a fatality rate of 3·77 per cent but one of these deaths was from sub-acute nephritis (lasting only six weeks) and occurred four months after delivery. The victim had not quite recovered from her anæmia when she commenced her terminal illness.

On both clinical and hæmatological grounds we may say that the microcytic-hypochromic anæmia associated with pregnancy is probably identical with that seen in non-pregnant women. It is a comparatively mild disease and does not seem to have any direct relationship with malaria but it may have some connection with hookworm infection since the incidence of heavy infections is comparatively high in this group. The arguments advanced in Part V against hookworm infection being the sole cause apply equally to pregnant as to non-pregnant women.

NORMOCYTIC-HYPO-ORTHOCHROMIC GROUP.

This group has not been described as a separate entity by other authors but the different appearance of the stained blood films of these cases from the appearance of films from the other groups is so marked that they must be classified separately. The group may be classed as normocytic as the range and mean of values of M. C. V. are roughly equivalent to what might be expected in a series of healthy Europeans.

The distribution was also remarkably central—

1 case had value lower than   85·0 cubic microns.
5 ,, ,,   between   85·0 and 94·99   cubic microns.
5 ,, ,, ,,   95·0 ,,   104·99 ,,
2 ,, ,, ,,   105·0 ,,   114·99 ,,

As compared with the 'normal' coolies, of course, the group as a whole is macrocytic. Half the cases could be described as being hypochromic and the others as orthochromic—

In 2 cases the M. C. H. was between 15·0 and 18·99 micro-micrograms.
In 4 ,, ,, ,,   19·0 ,,   22·99 ,,
In 3 ,, ,, ,,   23·0 ,,   26·99 ,,
In 6 ,, ,, ,,   27·0 ,,   30·99 ,,
In 1 ,, ,,   higher than 31·0 micro-micrograms.

Saturation of the red cells was deficient in three-quarters of the group. Apart from the higher values of M. C. V. the chief point of difference between this group and the preceding one lies in the lower red cell counts found in this group. No less than 81 per cent of the counts were between 2·0 and 2·99 millions. The range and means of hæmoglobin values and of white cell counts are very similar to those found in group 'A': the distribution of findings is also similar.

The proportion of histories of recent malaria, 12·5 per cent, was almost identical with that in group 'A' but splenomegaly was found more often, in 31 per cent.

A history of previous anæmia was rather more common and there was a rather higher incidence of hookworm infection. No death occurred in this group.

The evidence available suggests that this type of anæmia like the microcytic type is mild in its effects though the anæmia itself is of higher degree. The evidence as to malaria being a causal agent is conflicting. It cannot be merely a secondary anæmia from blood loss, such as is seen in a European after malaria, nor from blood dilution due to pregnancy as is seen in America because the normal condition of the coolie's red cells is microcytic. On the other hand I have never seen this type of anæmia in coolies apart from pregnancy which suggests that the presence of the fœtus is the exciting cause. I suggest that it is, in fact, a mild type of tropical macrocytic anæmia without any hæmolytic element due to a slight defect of the vitamin-B complex in the diet. I suggest further that the defect is only slight because it is a 'conditioned defect', that is to say, the needs of the fœtus are sufficient to turn a minimal intake by the mother into an actual lack.

Micro-macrocytic-hypo-orthochromic group.

This group probably corresponds with the 'normocytic' group of other workers, or rather with the greater proportion of that group but it is considered that, from the point of view of cell volume, the term I have adopted is better since the range of values of M. C. V. is greater than would be expected in a group of healthy Europeans and is greater than that found in group 'B'. Moreover the distribution was much more scattered—

11 cases had values between 85·0 and 94·99 cubic microns.
6 ,, ,, ,, 95·0 ,, 104·99 ,,
3 ,, ,, ,, 105·0 ,, 114·99 ,,
2 ,, ,, . ,, 115·0 ,, 124·99 ,,
2 ,, ,, higher than 125·0 cubic microns.

The degree of anisocytosis in the stained films was also sufficiently remarkable to call for comment: such large numbers of both large and small cells were so constantly seen that the M. C. V. does not give a true picture of the condition of the erythrocytes. I do not consider that to split this group into two and call all cases with M. C. V. over 95 cubic microns macrocytic would be a wise move as the range and distributions of M. C. H. values in the two groups would be similar and the characteristics of the stained films would be identical. A similar argument applies against any sub-division of this group based on M. C. H. values. Nucleated red cells were seen in nine cases. The means of both red cell counts and hæmoglobin content are rather lower than those in the microcytic group, indicating a more severe anæmia. In no case was either the M. C. H. or M. C. H. C. greater than thirty, indicating incomplete saturation of the red cells and no tendency to a true hyperchromic anæmia. The distribution of values of M. C. H., M. C. H. C., red cell counts and white cell counts was, in each case, widely scattered about the mean. On these findings I have adopted the name micro-macrocytic-hypo-orthochromic anæmia and regard it as being analogous with the type seen in the smaller

group of the non-pregnant series, though relatively more frequently seen in pregnant women.

All degrees of age, parity and residence in Assam were represented in this group. The proportion of those who had previously been anæmic was low, 12 per cent and so was the incidence of heavy hookworm infections, 26 per cent. On the other hand, 40 per cent had recently suffered from malaria and 32 per cent had an enlarged spleen. Œdema of the extremities was common, 44 per cent, a much higher proportion than in either of the two preceding groups. Three women in this group died, a fatality rate of 12 per cent so that this type of anæmia can be described as severe and frequently fatal.

The available evidence suggests that it is not associated with hookworm infection but that it does bear a relationship to malaria. Hæmatologically it is a mixed anæmia having both macrocytic and microcytic elements, each presumably due to a different cause but I do not consider that it can fairly be regarded simply as a mixture of two different types. Napier and Majumdar (*loc. cit.*) have discussed the possible dietetic deficiencies responsible for both microcytic and macrocytic types of anæmia and Fairley *et al.* (*loc. cit.*) have also pointed out that in many cases of tropical macrocytic anæmia there is a hæmolytic element due to chronic malaria. Following this reasoning I consider that this type of anæmia is produced by the interaction of the following three conditions, all present at the same time :—

(*a*) Deficiency of minerals, especially iron, in the diet,

(*b*) Deficiency of the vitamin-B complex in the diet,

(*c*) Excessive hæmolysis due to chronic malaria.

Since this type of anæmia was seen apart from pregnancy and since, in spite of an obvious iron deficiency (shown by a low value of M. C. H.), many of these cases still had a very high M. C. V. I think it probable that the *dietary defects* in this group are absolute rather than conditioned, in contra-distinction to the suggestion put forward when considering group 'B'.

MACROCYTIC-HYPERCHROMIC GROUP.

The means of the hæmoglobin estimations and of the red cell counts in this group are markedly lower than those in any of the preceding three groups : in neither case, however, is the distribution symmetrical about the mean. The means are artificially low because of a few very low values. The mean and distribution of leucocyte counts are within normal limits. In all cases where the necessary investigations were done, the M. C. V. was over 120 cubic microns and the M. C. H. over 30 micro-micrograms. The distribution of M. C. H. C. values was as follows :—

1 case had value	lower than	25·0	per cent.	
3 ,, ,,	between	25·0 and 30·9	per cent.	
1 ,, ,,	higher than	31·0	per cent.	

They were, therefore, all true cases of macrocytic-hyperchromic anæmia, the saturation of the red cells with hæmoglobin being within normal limits. All the stained films of this group showed obvious megalocytosis together with the presence of nucleated red cells, both normoblasts and megaloblasts being present in all cases.

Seven, or 54 per cent of this group, had previously suffered from anæmia. A similar number had recently had malaria and a like number had enlarged spleens. Œdema of the extremities was noted in 46 per cent. All these proportions are higher than the corresponding figures for the whole series. Of those whose stools were examined, 44 per cent were found to be heavily infected. Three of the thirteen women in this group died, a fatality rate of 23·1 per cent, easily the heaviest mortality in the whole series.

The anæmia encountered in this group is obviously of a very severe and fatal type : on clinical and hæmatological grounds it appears to be identical with the nutritional macrocytic anæmia of hæmolytic type found in Macedonia and described by Fairley *et al.* (*loc. cit.*). It also corresponds with the type found in the three hyperchromic and two of the orthochromic cases in the series of 57 pregnant anæmics discussed by Napier and Majumdar (*loc. cit.*). To my mind it is unfortunate that these two authors failed to separate these cases from the rest of their ' liver-Marmite group ' as it seems to me that these are the cases which are the most likely to succumb and, therefore, require the most vigorous investigation. In my experience the vast majority of cases of anæmia in pregnancy do perfectly well without any treatment, recovering completely after delivery so that, in order to ensure adequate treatment to those who really require it, some simple method of diagnosing the severe type is required.

Undetermined group.

The range of values of hæmoglobin in this group was from 2·0 g. to 7·3 g. per 100 c.c. of blood, the mean being 5·52 g. The distribution was somewhat eccentric as follows :—

10 cases had values between 2·0 and 3·9 grammes.
31   ,,   ,,   4·0 ,, 5·9   ,,
34   ,,   ,,   6·0 ,, 7·9   ,,

The incidence of previous anæmia, recent malaria and splenomegaly was, in each instance, almost identical with that for the whole series. Œdema was noted in 47·4 per cent compared with 36·8 for the whole series and the fatality rate in this group was 6·6 per cent against 7·1 per cent in the whole series. These findings, together with the nature of the distribution and the mean of the hæmoglobin estimations, are sufficient to justify the assumption that this group is comparable to the whole series and that, had further investigations been carried out, it would have resolved itself into the same four hæmatological groups in similar numerical proportions.

Further consideration of fatal cases.

The length of residence in Assam did not appear to have any bearing on prognosis nor did the fatal cases predominate in any particular age group or degree of parity. Half of those who died gave a history of recent malaria and half, also, had enlargement of the spleen while œdema of the extremities was seen in three-quarters. No prognostic significance was found to attach to cardiac enlargement nor to abnormal cardiac sounds nor to pulse rates. Though some of these cases showed a high degree of anæmia, seven, or just over half, had between 5·0 and 6·5 grammes of hæmoglobin per 100 c.c., so that it is difficult to claim any prognostic value for simple hæmoglobin readings.

I suggest that, in the absence of laboratory facilities, the danger signals in a case of anæmia in pregnancy are splenomegaly, œdema of the extremities, and a history of recent malaria, and that the co-existence of any two of these signs should be an indication for intensive treatment. To these signs, where facilities exist for examining stained blood smears, might be added the presence of nucleated red cells since these were frequently found in the two groups with the heaviest fatality rates.

CONCLUSIONS.

The assumption that the entire coolie population of the tea estates of Assam is, by Western standards, anæmic, is borne out by the study of the hæmoglobin values of 80 coolies reported in Part II of this paper and a further study of these values in young children suggested that the anæmic state is congenital and is not the result of immigration into Assam. This suggestion was borne out by the results of examination of new recruits (reported in Part III) who were found to present a partially anæmic state almost identical with that seen in the settled population. The comparative universality of the condition suggests that its basic cause is something even more widespread than either malaria or hookworm infection. Again, since the common severe microcytic anæmia consists, essentially, in a reduction in the number of red cells together with a fairly proportional reduction in the amount of hæmoglobin, without any further gross disturbance of the erythron, it is probable that its cause is simply an exacerbation of the original, permanent underlying cause of the permanent mild anæmia. This supports the theory that a more widespread cause than either malaria or hookworm infection is responsible and that some long-standing defect in the diet is at the root of the trouble.

The above remarks apply only to the common anæmia met with in all classes of coolies apart from pregnancy. When the anæmias complicating pregnancy are considered, a new feature appears for, while the anæmia in the majority of cases is of the microcytic-hypochromic type, quite a large proportion are found to have a tendency towards the macrocytic-hyperchromic type. In these cases particularly, there is evidence that malaria plays a part in the causation, bearing out the observations of other workers both in India and in Europe. Until evidence that any large proportion of these macrocytic anæmias are non-hæmolytic in type or that this hæmolytic type occurs in areas where malaria does not

exist, I think we must assume that their ætiology is different from that of the common anæmia and that they are produced by an interaction of dietetic deficiency, the presence of the fœtus and chronic malaria acting on an already inefficient hæmopoietic system.

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· *Microcytic-hypo*

Serial number.	Age in years.	Assam residence in years.	Parity.	Previously anæmic ?	Recently had malaria ?	Is spleen enlarged ?	Is liver enlarged ?	Abnormalities in lungs ?	Is œdema present ?	Is heart enlarged ?	Cardiac sounds.	Pulse rate per minute.	Urine.
1	35	12	5	No	No	Yes	No	No	No	No	n	84	n
2	30	30	5	No	No	No	No	No	No	No	HB	90	n
3	18	1	1	No	No	No	No	No	No	No	HB	104	n
4	20	1	2	No	No	No	No	No	No	No	n	96	n
5	30	5	5	No	No	No	No	No	No	No	n	82	n
6	24	4	2	No	No	No	No	No	No	No	n	80	n
7	30	20	4	No	No	No	No	No	No	No	n	86	n
8	30	1	4	No	No	Yes	No	No	No	No	PS. HB	80	TA
9	26	8	4	No	No	No	No	No	No	No	Sy	118	n
10	18	18	2	Yes	No	No	No	No	No	No	HB	100	A
11	28	5	4	Yes	No	No	No	Ra	No	No	HB	100	*
12	20	1	1	No	No	No	No	No	No	No	n	120	n
13	30	2	6	No	No	No	No	No	No	No	n	90	n
14	32	18	7	Yes	No	No	No	No	No	No	n	78	n
15	22	1	2	No	No	No	No	No	No	No	n	110	n
16	30	3	4	No	Yes	No	No	No	No	No	n	100	n
17	30	20	4	No	No	No	No	Ra	No	No	n	100	n
18	23	21	2	No	No	No	No	Ra	Yes	No	n	88	n
19	28	21	4	No	No	No	No	Ra	No	No	HB	110	n

? = Examination not done.
Ra = Râles in lungs.
n = Normal.
HB = Hæmic bruit.

PS = Accentuation of pulmonary second sound.
Sy = Mitral systolic murmur.
TA = Trace of albumin.
A = Albuminuria.

COL.

Stool.			Hæmoglobin in g. per 100 c.c.	R. B. C. in millions per c.mm.	Leucocytes per c.mm.	M. C. H. in micro-micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	Nucleated reds in film.	Result.
Hookworm.	Ascaris.	Trichuris.								

chromic group.

L	..	L	4·0	3·06	10,000	13·07	69·44	18·82	..	Recovery.
L	L	..	5·8	3·94	8,000	14·72	71·93	20·46	..	do.
H	..	..	5·6	3·26	6,000	17·18	75·23	22·83	..	do.
..	..	..	6·8	4·05	3,200	16·79	77·77	21·58	..	do.
L	..	..	4·8	2·47	9,800	19·39	81·62	23·76	..	do.
H	..	M	5·2	4·18	11,200	12·48	62·58	19·62	..	do.
L	..	..	6·4	3·30	4,200	19·39	77·61	24·99	..	do.
L	..	..	6·3	4·05	3,000	15·55	63·28	23·12	..	do,
L	..	..	7·0	3·59	7,400	19·64	68·30	28·50	..	do.
M	..	L	4·8	2·86	9,600	16·77	81·57	20·48	..	do.
H	..	..	5·6	2·80	6,400	20·00	81·75	24·46	..	Died four months after delivery from nephritis.
M	..	..	4·6	3·05	7,400	15·08	75·05	20·09	..	Recovery.
L	..	L	6·2	4·53	5,400	13·68	56·52	24·20	..	do.
L	L	..	9·0	4·88	5,200	18·44	71·47	25·80	..	do.
H	..	..	6·9	3·32	8,600	20·79	82·07	25·32	..	do.
H	M	..	6·9	3·34	6,800	20·66	81·50	25·32	..	do.
M	..	..	6·7	4·11	5,200	16·30	64·96	25·09	..	do.
M	..	L	4·7	2·24	7,600	22·77	80·26	26·14	..	do.
M	..	..	7·8	4·45	8,800	17·52	68·58	25·55	..	do.

* = No albumin at time of examination but developed nephritis later.
H = Heavy infection.
M = Moderate infection.
L = Light infection.

Serial number.	Age in years.	Assam residence in years.	Parity.	Previously anæmic ?	Recently had malaria ?	Is spleen enlarged ?	Is liver enlarged ?	Abnormalities in lungs ?	Is œdema present ?	Is heart enlarged ?	Cardiac sounds.	Pulse rate per minute.	Urine.
20	22	12	4	Yes	No	No	No	No	No	No	n	100	n
21	28	20	6	No	Yes	Yes	No	No	No	No	HB	86	n
22	23	2	2	Yes	No	No	No	No	No	No	HB	100	n
23	18	1	1	No	Yes	No	No	No	No	No	Sy	110	n
24	28	1	2	No	No	No	No	No	Yes	No	HB	90	n
25	24	24	1	Yes	No	No	No	No	Yes	No	HB	98	n
26	17	2	2	No	Yes	No	No	No	No	No	HB	102	n
27	32	22	5	Yes	No	No	No	No	No	No	HB	90	n
28	20	1	1	No	No	No	No	No	Yes	No	PS	76	n
29	25	2	2	No	No	No	No	No	No	No	HB	96	n
30	17	3	1	Yes	No	No	No	No	No	No	n	112	n
31	24	$\frac{1}{2}$	2	No	No	No	No	No	Yes	No	n	102	TA
32	14	14	1	No	Yes	Yes	No	No	No	No	n	94	TA
33	30	30	5	No	No	No	No	No	Yes	No	n	88	n
34	38	$\frac{1}{2}$	3	No	No	No	No	No	Yes	No	n	76	n
35	30	20	6	No	No	No	No	No	No	No	PS	120	n
36	20	12	3	Yes	No	No	No	No	No	No	HB	120	n
37	22	10	3	Yes	No	No	No	No	No	No	n	88	n
38	26	5	Mp	No	No	No	No	No	No	Yes	HB	90	n
39	30	4	1	No	No	No	No	No	Yes	No	n	90	n
40	23	7	2	No	No	No	No	No	No	No	n	84	n

? = Examination not done.
n = Normal.
HB = Hæmic bruit.
Sy = Mitral systolic murmur.

PS = Accentuation of pulmonary second sound.
Mp = Multipara.
TA = Trace of albumin.

COL.

Stool.			Hæmoglobin in g. per 100 c.c.	R. B. C. in millions per c.mm.	Leucocytes per c.mm.	M. C. H. in micro-micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	Nucleated reds in film.	Result.
Hookworm.	Ascaris.	Trichuris.								
H	..	..	5·6	3·84	7,200	14·58	70·96	20·55	..	Recovery.
L	..	..	4·4	2·84	10,000	15·49	70·98	21·82	..	do.
H	..	..	3·8	2·54	7,400	14·96	84·82	17·64	..	do.
M	..	..	5·0	3·28	10,800	15·24	68·12	22·38	..	do.
M	..	..	4·6	3·07	8,000	14·98	76·33	19·63	..	do.
H	..	..	3·4	3·08	8,000	11·04	70·05	15·99	..	do.
..	..	..	7·2	4·03	6,600	17·87	71·67	24·92	..	do.
H	..	..	6·0	4·60	7,200	13·04	68·72	18·98	..	do.
H	..	..	4·4	2·86	4,200	15·38	76·22	20·18	..	do.
?	?	?	4·4	3·43	4,200	12·85	73·09	17·55	..	do.
L	..	..	6·0	3·73	8,000	16·08	77·42	20·79	..	do.
..	..	..	5·8	3·85	5,600	15·06	76·44	19·71	..	do.
H	H	M	6·6	3·48	7,600	18·96	76·72	24·64	..	do.
L	L	L	7·2	4·26	8,000	16·90	72·91	23·18	..	do.
..	..	..	7·2	3·96	16,000	18·18	77·07	23·59	..	do.
L	..	..	6·4	3·57	13,200	17·93	77·86	23·02	..	do.
M	..	L	4·0	2·56	5,400	15·60	78·77	19·84	..	do.
M	H	L	6·0	2·91	7,400	20·61	?	?	..	do.
H	..	H	6·3	2·63	4,800	23·95	?	?	..	do.
L	L	..	6·5	5·14	11,200	12·64	?	?	..	Developed anasarca two weeks after delivery and died in three days.
M	..	..	7·5	3·25	8,400	23·07	?	?	..	Recovery.

? = Examination not done.
M = Moderate infection.

L = Light infection.
H = Heavy infection.

Serial number.	Age in years.	Assam residence in years.	Parity.	Previously anæmic ?	Recently had malaria ?	Is spleen enlarged ?	Is liver enlarged ?	Abnormalities in lungs ?	Is œdema present ?	Is heart enlarged ?	Cardiac sounds.	Pulse rate per minute.	Urine.
41	21	2	1	No	No	No	No	No	No	Yes	HB	90	n
42	18	3	2	Yes	No	No	No	No	No	No	n	120	n
43	35	½	5	No	No	No	No	No	No	No	PS	120	n
44	22	20	2	No	No	No	No	No	No	No	n	90	n
45	35	26	7	No	No	No	No	No	No	No	HB	130	n
46	24	2	2	No	No	No	No	No	No	No	PS. HB	80	n
47	18	5	2	No	No	No	No	No	No	No	HB	130	n
48	25	6	3	No	No	No	No	No	No	No	n	78	n
49	28	22	3	No	Yes	Yes	No	No	No	No	HB	80	n
50	18	1	1	No	Yes	Yes	No	No	Yes	No	PS. HB	130	A
51	29	5	4	No	No	No	No	No	No	No	n	96	n
52	33	½	5	No	No	No	No	No	Yes	No	n	110	n
53	26	26	3	No	No	No	No	No	No	No	n	120	n

Normocytic-hypo-

54	27	3	3	No	No	Yes	No	No	No	No	n	72	n
55	28	26	3	No	No	Yes	No	No	Yes	No	n	108	n
56	40	35	9	Yes	No	No	No	No	No	No	n	84	n
57	16	16	1	Yes	Yes	Yes	No	No	No	No	HB	110	n
58	26	18	4	No	No	No	No	No	No	No	HB	92	TA
59	28	6	3	No	No	No	No	No	No	No	n	96	n
60	27	2	1	Yes	No	No	No	No	No	No	PS. HB	80	n

? = Examination not done.   PS = Accentuation of pulmonary
HB = Hæmic bruit.   second sound.
n = Normal.   TA = Trace of albumin.
A = Albuminuria.

COL.

STOOL.			Hæmoglobin in g. per 100 c.c.	R. B. C. in millions per c.mm.	Leucocytes per c.mm.	M. C. H. in micro- micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	Nucleated reds in film.	RESULT.
Hookworm.	Ascaris.	Trichuris.								
H	..	L	7·5	4·29	11,800	17·48	?	?	..	Recovery.
H	H	..	6·8	3·32	8,000	20·48	?	?	..	do.
M	..	..	4·3	2·14	9,600	20·09	?	?	..	do.
H	..	..	7·5	4·25	9,400	17·64	?	?	..	do.
M	M	L	7·0	3·67	8,400	19·07	?	?	..	do.
M	..	..	8·2	4·42	6,400	18·55	?	?	..	do.
L	..	L	7·0	4·01	9,600	17·45	?	?	..	do.
L	..	..	6·7	3·52	6,200	19·17	?	?	..	do.
L	L	..	6·8	3·80	11,600	17·89	?	?	..	do.
H	M	..	3·8	2·45	11,600	15·51	?	?	..	do.
M	..	..	6·8	3·59	6,400	18·94	?	?	..	do.
H	..	..	3·6	2·29	?	15·68	?	?	..	do.
H	H	H	6·6	4·55	12,000	14·50	?	?	..	do.

orthochromic group.

M	..	M	6·4	2·59	9,200	24·71	103·08	23·97	N	do.
L	L	..	6·2	2·01	3,000	30·84	83·05	35·55	..	do.
L	..	..	6·6	2·39	7,200	27·61	88·80	31·05	..	do.
?	?	?	6·2	2·37	10,600	26·16	110·38	23·70	..	do.
H	..	..	5·6	2·78	5,400	20·14	105·84	19·03	..	do.
H	..	L	6·0	2·88	6,600	20·82	94·60	22·02	..	do.
H	..	..	6·3	2·14	8,000	29·44	96·70	30·42	..	do.

? = Examination not done.   M = Moderate infection.
H = Heavy infection.   L = Light infection.

Serial number.	Age in years.	Assam residence in years.	Parity.	Previously anæmic ?	Recently had malaria ?	Is spleen enlarged ?	Is liver enlarged ?	Abnormalities in lungs ?	Is œdema present ?	Is heart enlarged ?	Cardiac sounds.	Pulse rate per minute.	Urine.
61	35	25	10	No	No	No	No	No	No	No	HB	92	n
62	22	½	2	No	No	No	No	No	No	No	n	82	n
63	25	2	1	No	No	No	No	No	No	No	n	70	n
64	14	12	1	No	No	Yes	No	No	Yes	No	HB	80	n
65	28	½	3	No	No	Yes	No	No	Yes	No	n	72	n
66	21	21	1	Yes	No	No	No	No	Yes	Yes	HB	89	n
67	27	1	2	No	No	No	No	No	No	No	n	110	n
68	30	23	7	No	Yes	No	No	Ra	No	No	n	130	n
69	22	2	3	No	No	No	No	No	No	No	HB	102	n

Micro-macrocytic-hypo·

Serial number.	Age in years.	Assam residence in years.	Parity.	Previously anæmic ?	Recently had malaria ?	Is spleen enlarged ?	Is liver enlarged ?	Abnormalities in lungs ?	Is œdema present ?	Is heart enlarged ?	Cardiac sounds.	Pulse rate per minute.	Urine.
70	22	22	3	Yes	Yes	Yes	Yes	No	No	Yes	PS. HB	122	n
71	25	10	6	Yes	Yes	No	Yes	No	No	No	n	94	n
72	23	½	3	No	Yes	Yes	No	No	Yes	Yes	Sy	110	n
73	18	18	2	Yes	Yes	No	No	No	Yes	Yes	n	100	TA
74	30	22	6	No	Yes	Yes	No	No	Yes	No	PS. HB	124	n
75	30	½	4	No	Yes	No	No	No	No	No	n	90	?
76	22	2	2	No	No	No	No	No	Yes	No	HB	100	n
77	38	3	7	No	No	Yes	No	No	No	No	HB	92	A

? = Examination not done.
Ra = Râles in lungs.
HB = Hæmic bruit.
n = Normal.

PS = Accentuation of pulmonary
     second sound.
Sy = Mitral systolic murmur.
TA = Trace of albumin.

COL.

STOOL.			Haemoglobin in g. per 100 c.c.	R. B. C. in millions per c.mm.	Leucocytes per c.mm.	M. C. H. in micro-micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	Nucleated reds in film.	RESULT.
Hookworm.	Ascaris.	Trichuris.								
L	..	..	5·6	2·60	6,600	21·53	96·42	22·33	..	Recovery.
L	..	..	7·8	3·19	7,000	25·08	104·22	23·46	..	do.
H	..	..	4·8	2·90	6,200	16·55	86·45	19·15	..	do.
H	M	M	3·8	2·16	6,400	17·59	95·88	13·52	..	do.
M	H	..	7·2	3·66	3,000	19·67	89·34	22·02	..	do.
H	..	..	4·2	1·32	3,600	31·82	90·83	35·03	..	do.
L	..	..	6·3	2·20	7,400	28·64	?	?	..	do.
L	H	..	6·7	2·42	9,000	27·68	?	?	..	do.
L	L	..	6·0	2·16	9,200	27·77	?	?	..	do.

orthochromic group.

L	..	..	6·4	2·18	5,200	29·36	110·00	26·79	N	do.
..	..	..	5·2	2·59	4,800	20·08	92·59	21·68	..	do.
?	?	?	4·4	2·09	5,400	21·05	91·24	23·07	..	do.
L	..	..	4·6	1·75	8,000	26·28	99·66	26·38	N. Mg	do.
H	H	H	4·2	2·20	12,400	19·10	111·48	17·13	N. Mg	do.
?	?	?	3·8	1·66	?	22·89	147·74	15·49	N. Mg	do.
..	M	L	3·8	1·60	8,600	23·75	112·41	21·68	..	Died in premature (35 weeks) labour, undelivered.
H	L	L	4·4	2·46	5,800	17·88	88·62	20·18	..	Developed acute enteritis six weeks after delivery and died.

A = Albuminuria.
L = Light infection.
H = Heavy infection.

M = Moderate infection.
N = Normoblasts.
Mg = Megaloblasts.

J, MR

Serial number.	Age in years.	Assam residence in years.	Parity.	Previously anæmic ?	Recently had malaria ?	Is spleen enlarged ?	Is liver enlarged ?	Abnormalities in lungs ?	Is œdema present ?	Is heart enlarged ?	Cardiac sounds.	Pulse rate per minute.	Urine.
78	20	20	1	No	Yes	Yes	Yes	No	Yes	Yes	HB	84	n
79	28	4	3	No	No	No	No	No	Yes	No	n	84	n
80	24	¼	2	No	No	No	No	No	Yes	No	n	120	A
81	26	2	1	No	No	No	No	No	Yes	Yes	HB	120	n
82	29	3	3	No	No	Yes	No	No	Yes	Yes	n	96	n
83	26	20	3	No	No	No	No	No	Yes	Yes	HB	96	n
84	28	20	4	No	No	No	No	No	No	No	n	78	n
85	18	2	2	No	No	No	No	No	No	No	HB	80	n
86	30	1	6	No	Yes	Yes	No	No	No	No	n	100	n
87	18	18	2	No	No	No	No	No	No	No	n	96	n
88	35	20	8	No	No	No	No	No	No	No	n	78	n
89	22	6	4	No	No	No	No	No	No	No	n	84	n
90	18	1	1	No	No	No	No	No	No	No	HB	90	n
91	21	¼	1	No	No	Yes	No	No	Yes	Yes	HB	92	n
92	22	22	3	No	Yes	No	No	No	No	No	n	82	n
93	16	16	1	No	Yes	No	No	No	No	No	n	80	n
94	24	½	1	No	No	No	No	No	No	No	n	120	n

Macrocytic-hyper

| 95 | 22 | 17 | 3 | No | Yes | Yes | No | No | Yes | Yes | Sy | 114 | n |
| 96 | 18 | 1 | 1 | No | No | Yes | No | No | Yes | Yes | n | 90 | n |

? = Examination not done.   Sy = Mitral systolic murmur.
HB = Hæmic bruit.   A = Albuminuria.
n   = Normal.

COL.

Stool.			Haemoglobin in g. per 100 c.c.	R. B. C. in millions per c.mm.	Leucocytes per c.mm.	M. C. H. in micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	Nucleated reds in film.	Result.
Hookworm.	Ascaris.	Trichuris.								
M	M	..	5·3	1·87	5,400	28·34	116·57	24·31	..	Died suddenly during full-term labour.
M	..	..	5·8	3·18	5,200	18·23	90·50	20·08	..	Recovery.
H	L	..	5·2	2·43	7,000	21·39	89·71	23·85	..	do.
L	..	..	3·8	1·74	6,600	21·84	100·23	21·79	..	do.
L	..	..	3·4	1·93	7,800	17·62	93·19	18·90	..	do.
..	L	..	4·0	1·70	3,000	23·53	118·59	19·84	N. Mg	do.
L	L	..	5·0	2·46	5,600	20·32	88·60	22·93	..	do.
M	..	L	4·8	2·63	11,400	18·25	99·46	18·34	..	do.
H	..	..	5·8	2·81	6,000	20·64	93·09	20·17	..	do.
L	L	..	7·5	2·85	8,800	26·31	87·96	29·91	..	do.
M	..	..	7·5	2·98	10,400	25·16	95·10	26·46	..	do.
M	L	..	6·4	2·27	6,800	28·19	103·24	27·31	..	do.
H	..	..	3·6	2·31	9,200	15·59	87·29	17·85	N. Mg	do.
L	L	..	3·8	1·46	7,200	26·03	97·05	26·81	N. Mg	do.
..	..	..	4·6	2·94	6,200	15·64	92·68	16·88	N	do.
..	..	..	6·2	2·09	13,400	29·66	135·59	21·87	N	do.
H	L	..	6·6	2·59	10,600	25·48	?	?	..	do.

chromic group.

L	..	..	5·2	1·40	4,600	37·14	140·14	26·50	N. Mg	do.
M	..	..	3·0	0·73	12,600	41·09	164·25	25·02	N. Mg	Suddenly developed dyspnœa and died at 39 weeks undelivered.

M = Moderate infection.
L = Light infection.
H = Heavy infection.

N = Normoblasts.
Mg = Megaloblasts.

Serial number.	Age in years.	Assam residence in years.	Parity.	Previously anæmic ?	Recently had malaria ?	Is spleen enlarged ?	Is liver enlarged ?	Abnormalities in lungs ?	Is œdema present ?	Is heart enlarged ?	Cardiac sounds.	Pulse rate per minute.	Urine.
97	25	25	2	Yes	No	Yes	No	No	Yes	No	HB	84	n
98	30	½	5	No	No	No	No	No	No	Yes	n	106	n
99	25	25	5	Yes	Yes	Yes	No	No	No	No	HB	113	TA
100	32	9	5	No	Yes	Yes	Yes	No	No	No	n	80	n
101	30	10	5	No	No	No	No	No	No	No	n	84	n
102	24	7	Mp	No	No	Yes	No	No	Yes	Yes	HB	90	n
103	28	20	4	Yes	Yes	Yes	No	No	No	No	HB	90	n
104	19	3	1	Yes	No	No	No	No	No	No	n	80	n
105	25	20	4	Yes	Yes	No	No	No	Yes	Yes	HB	104	?
106	18	5	2	Yes	Yes	No	No	Rh	No	Yes	HB	92	?
107	35	35	6	Yes	Yes	No	No	Rh	Yes	No	PS. HB	92	?

? = Examination not done.
Mp = Multipara.
Rh = Rhonchi in lungs.
HB = Hæmic bruit.

PS = Accentuation of pulmonary second sound.
n = Normal.
TA = Trace of albumin.

COL.

Stool.			Haemoglobin in g. per 100 c.c.	R. B. C. in millions per c.mm.	Leucocytes per c.mm.	M. C. H. in micro-micrograms.	M. C. V. in cubic microns.	M. C. H. C. in per cent.	Nucleated reds in film.	Result.
Hookworm.	Ascaris.	Trichuris.								
H	L	L	4·8	1·41	6,400	34·04	139·15	24·46	N. Mg	Recovery.
..	L	..	5·8	1·59	6,800	36·48	137·11	26·60	N. Mg	do.
L	M	M	6·7	1·71	3,200	39·18	121·11	32·35	N. Mg	do.
H	H	H	6·2	1·81	6,000	34·25	?	?	N. Mg	Developed pneumonia and jaundice t e n d a y s after delivery and died in 24 hours.
H	H	M	6·3	1·75	5,400	36·00	?	?	N. Mg	Recovery.
H	..	L	6·1	1·87	4,400	32·62	?	?	N. Mg	do.
L	..	L	6·9	1·46	7,400	47·26	?	?	N. Mg	do.
?	?	?	3·5	1·00	9,600	35·00	?	?	N. Mg	do.
?	?	?	4·8	?	?	?	?	?	N. Mg	do.
?	?	?	5·8	?	?	?	?	?	N. Mg	do.
?	?	?	1·9	?	?	?	?	?	N. Mg	Died undelivered with s y m p t o m s of air hunger.

H = Heavy infection.
M = Moderate infection.
L = Light infection.

N = Normoblasts.
Mg = Megaloblasts.

NORMAL HÆMATOLOGICAL STANDARDS IN THE MONKEY (*MACACUS SINICUS*).

BY

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WILLS and her co-workers (Wills and Bilimoria, 1932; Wills and Stewart, 1935) have reported the development of a nutritional macrocytic-hyperchromic anæmia in monkeys fed on diets resembling those consumed by patients suffering from 'tropical macrocytic anæmia' in Bombay. An experiment is at present in progress in the Laboratories in which the hæmatological and other changes occurring in monkeys fed on ill-balanced rice diets are being investigated. In order to provide a standard of comparison for the hæmatological findings in the deficient animals, the blood in a series of normal monkeys fed on a well-balanced and liberal diet has been studied.

MATERIAL AND METHODS OF INVESTIGATION.

Thirteen *Macacus sinicus* monkeys (group A), varying in weight from 4 lb. to 10 lb., were fed on a diet based on whole wheat, milk, and vegetables, resembling that given to the stock rats in the Coonoor Laboratories. Its composition was as follows :—

	Per monkey per day.
Atta (whole-wheat flour)	.. 100 g.
Pulse	.. 15 g.
Whole milk ..	.. 150 c.c.
Butter	.. 10 g.
Root vegetables	.. 40 g.
Other raw vegetables	.. 40 g.
Fruit	.. 20 g.

The atta, pulse, and butter were given together in the form of a 'chapatti' (unleavened cake). The other foods were fed separately in the raw state. The kind of pulses, vegetables, and fruit supplied were varied from day to day.

The animals were kept in separate cages under thoroughly hygienic conditions. They were housed in airy rooms, the temperature of which was kept fairly constant, by artificial heating, if necessary, during the cold season. The animals were set free for exercise every day in enclosed 'runs', which provide plenty of room and sunshine. The blood examinations were made after the animals had been in the Laboratories for about a year.

Methods of investigation.—The blood for red and white cell counts, smear and hæmoglobin estimation was non-oxalated and taken direct from puncture of the marginal vein of the ear. The blood for cell volume was obtained by venepuncture from the superficial vein on the posterior aspect of the leg and was oxalated in Wintrobe's dry oxalate mixture tubes (Hynes, 1939). These tubes are prepared by placing 0·1 ml. of a solution of 0·2 per cent potassium oxalate and 0·3 per cent ammonium oxalate in a small-sized tube and drying in an incubator, and one tube is used for every 0·25 ml. of blood.

The red and white cell counts were made in a new quadruple counting chamber (Levy-Hausser) * made of green glass with improved Neubauer and Fuchs-Rosenthal rulings. The main advantages of this slide are :—

(i) Two ruled separate chambers are provided for both red and white cell counts to facilitate making duplicate counts for greater accuracy,

(ii) The Fuchs-Rosenthal ruling (consisting of sixteen 1 mm. squares bordered by triple lines, with each 1 mm. square sub-divided into sixteen smaller squares by single lines) permits of great accuracy in the enumeration of leucocytes and minimizes the experimental error.

Toisson's fluid has been used for diluting red blood cells and Türk's solution for leucocytes. The counts were made immediately after dilution to prevent any lysis. The cell volume was estimated with the hematocrit. The hematocrit tubes were filled with blood and centrifugalized for 15 minutes, at about 2,500 revolutions per minute in the first instance, and for another five minutes subsequently to ensure that the maximum amount of packing of corpuscles had taken place.

The red cell diameter was measured by a modified direct method described by Sankaran and Radhakrishna Rao (1938). Two hundred and fifty cells were measured for each animal, and from the figures obtained the mean diameter was calculated.

The hæmoglobin was estimated with a Hellige Normal Hæmometer containing two non-fading coloured prisms for comparison. Square-shaped measuring tubes with Sahli scale were used for the blood solution and readings were taken one hour after the addition of blood to deci-normal hydrochloric acid.

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* Supplied by Arthur H. Thomas Co., Philadelphia, U.S.A.

RESULTS.

Tables I and II give the results. In order to study the possible effect of altitude on the blood, Coonoor being situated about 6,000 feet above mean sea-level, 26 animals (group B) were investigated soon after their arrival in the Laboratories from the plains. All these animals were obtained from a dealer in Madras city shortly after they were caught in the wild state. Hæmatological findings for these animals are incorporated in Tables I and II. Figures reported by other workers are included in the tables for purposes of comparison.

DISCUSSION.

The mean red cell count of the animals in group A was slightly higher, while the mean value for hæmoglobin was lower, than the figures for human beings given by Sokhey *et al.* (1937). The mean red cell diameter was close to that observed in a group of South Indians, which was 6·85 microns (Sankaran and Radhakrishna Rao, *loc. cit.*). Both the total and differential counts of leucocytes showed great variations. The mean total white cell count was much higher than that found in normal human beings. A preponderance of lymphocytes over polymorphonuclear cells was a constant feature in the differential counts.

There was no increase in the hæmoglobin level in the animals after being kept in Coonoor for about a year. In group A, the mean red cell count and the mean red cell diameter were slightly higher than in group B ; the difference in the case of the former was just significant statistically, but not significant in the case of the latter. Nucleated red cells were sometimes encountered in the peripheral blood of the animals during a short period after their arrival in Coonoor.

The literature on normal hæmatological standards in monkeys, except contributions by Klieneberger and Carl (1927) and Harmon *et al.* (1931), whose figures could not be obtained, is summarized in Tables I and II. In general, the results reported by these workers agree with those obtained by us.

SUMMARY.

A study of hæmatological standards in *Macacus sinicus* monkeys fed on a liberal diet based on wheat, milk, and vegetables has been undertaken. The following results were obtained :—

Total red cell count : 6·305 millions per cubic millimetre.
Hæmoglobin content (Sahli-Hellige scale) : 14·1 grammes per 100 ml.
Red blood cell diameter (modified Price-Jones' method) : 6·78 microns.
Volume of packed cells : 43·7 per cent.
Mean corpuscular volume : 70·54 cubic microns.
Mean corpuscular hæmoglobin : 21·09 micro-micrograms.
Mean corpuscular hæmoglobin concentration percentage : 32·54.

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VERDER and PETRAN (1937)	*Jour. Inf. Dis.*, **60**, p. 193.
WILLS and BILIMORIA (1932)	*Ind. Jour. Med. Res.*, **20**, p. 391.
WILLS and STEWART (1935)	*Brit. Jour. Exper. Path.*, **16**, p. 444.

TABLE I.

Hæmatological standards in monkeys.

Author.	Number of animals used.	Species of the monkey.	RED BLOOD CELLS — In millions per cubic millimetre of blood — Mean.	Standard deviation.	Co-efficient of variation.	Maximum.	Minimum.	Number of observations.	Diameter in microns — Mean.	Standard deviation.	Co-efficient of variation.	Maximum.	Minimum.	Number of observations.	HÆMOGLOBIN — In grammes per 100 ml. of blood — Mean.	Standard deviation.	Co-efficient of variation.
Present authors (1940), group A.	13	M. sinicus	**6·305**	0·806	12·78	8·025	4·00	38	**6·78**	0·18	2·6	7·04	6·44	13	**14·1**	1·53	10·88
Present authors (1940), group B.	26	,,	**5·69**	0·815	14·32	7·43	4·65	26	**6·55**	0·38	5·85	6·82	6·41	10	**14·1**	1·055	7·48
Hektoen and Eggers (1911).	4	M. rhesus															
Lucas and Prizer (1912).	4	,,															
Anderson and Neill (1915).	10	,,	4·6			6·1	3·6	20							10·2		
Taylor (1919)	40	,,															
Taylor et al. (1919)	4	,,													14·4		
Krumbhaar and Musser (1921).	13	,,	5·99			7·14	5·06	20									
Krumbhaar and Musser (1923).	7	,,	5·9			7·1	5·0	13							16·4		
Fox (1927)	..	,,	5·0			7·0	4·5										
Ponder (1928)	..	,,	4·9			5·5	4·5										
Hall (1929)	8	,,	5·59														
Scarborough (1931)	..	M. rhesus and others.															
Bilimoria (1931)	27	M. sinicus and rhesus.	6·43	0·65		7·88	5·17	27 ?	6·4	0·75		6·9	6·1	27 ?	12·53	2·61	
Wills and Stewart (1935).	13	M. rhesus	6·23		9·6	7·81	5·08	113	7·08			7·27	6·9	5	12·18		7·6 ?
Verder and Petran (1937).	3	,,	4·94														
Shukers et al. (1938)	19	M. mulatta	5·2			6·8	3·6	143							12·2		

TABLE 1—concld.

Author	Number of animals used	Species of the monkey	HÆMOGLOBIN—concld. In grammes per 100 ml. of blood			WHITE BLOOD CELLS — In thousands per cubic millimetre of blood						DIFFERENTIAL COUNT (PER CENT.)					
			Maximum	Minimum	Number of observations	Mean	Standard deviation	Co-efficient of variation	Maximum	Minimum	Number of observations	Polymorphonuclears	Lymphocytes	Eosinophiles	Large mononuclears	Mast cells	Number of observations
Present authors (1940), group A.	13	M. sinicus	19·03	11·07	86	**16·6**	4·53	27·3	26·52	10·00	22	**31·9** (11–64)	**62·0** (33–86)	**3·2** (0–20)	**2·7** (0–6)	**0·2** (0–1)	13
Present authors (1940), group B.	26	"	19·38	11·25	26	..	..	..	..	..	..	..	..	..	..	..	..
Hektoen and Eggers (1911).	4	M. rhesus	..	..	..	18·6	..	..	23·9	15·5	4	45 (24–65)	51	..	..	..	4
Lucas and Prizer (1912).	4	"	..	..	..	26·5	..	..	31·6	23·6	..	..	..	..	..	..	..
Anderson and Neill (1915).	10	"	11·3	8·7	20	11·2	..	..	12·6	8·3	20	42 (32–50)	54 (38–63)	..	..	..	20
Taylor (1919).	40	"	..	..	..	22·2	..	..	..	..	..	41·1	53·7	4·1	0·6	0·6	121
Taylor et al. (1919).	4	"	..	..	..	26·0	..	..	..	..	..	39 (34–44)	58 (54–63)	..	..	..	5
Krumbhaar and Musser (1921).	13	"	16·8	12·3	20	13·4	..	..	..	..	20	47	47	..	..	..	..
Krumbhaar and Musser (1923).	7	"	17·5	14·6	13	14·2	..	..	..	..	13	..	..	..	..	..	..
Fox (1927).	..	"	..	..	..	..	..	28·6	25·0	5·0	..	..	..	..	..	..	..
Ponder (1928).	..	"	..	..	..	10·4	..	..	16·0	7·0	..	(44–54)	(33–47)	..	..	..	..
Hall (1929).	8	"	14·42	10·5	..	14·2	..	..	20·5	7·5	..	43	52	..	..	..	..
Scarborough (1931).	..	M. rhesus and others.	..	..	..	16·21	..	..	..	..	..	..	..	..	..	..	..
Bilimoria (1931).	27	M. sinicus and rhesus.	13·80	11·17	27 ?	18·129	6·32	..	32·6	8·6	27 ?	35 (20–50)	61	2·0	2·0	..	27
Wills and Stewart (1933).	13	M. rhesus	15·12	10·36	107	15·24	..	28·6	28·7	8·2	116	41·5 (8–75)	52 (22–89)	2·1 (0–8)	2·4 (0–8)	..	57
Verder and Petran (1937).	3	"	..	..	..	14·58	..	..	..	..	..	38·2	59·7	1·0	0·8	..	27
Shukers et al. (1938).	10	M. mulatta	15·1	9·1	14·0	15·0	..	..	34·7	5·6	149	36 (7–81)	50 (14–89)	..	..	..	140

Note.—The figures in parentheses represent the minimum and maximum.

TABLE II.

Hæmatological standards in monkeys.

Author.	VOLUME OF PACKED CELLS. In percentage.					MEAN CORPUSCULAR HÆMOGLOBIN. In micro-micrograms ($\gamma\gamma$).				
	Mean.	Standard deviation.	Co-efficient of variation.	Maximum.	Minimum.	Mean.	Standard deviation.	Co-efficient of variation.	Maximum.	Minimum.
Present authors (1940), group A.	**43·7**	5·76	13·2	51	32	**21·09**	1·98	9·4	24·99	20·08
Present authors (1940), group B.	**49·7**	3·36	6·78	56	45	**24·69**	3·214	13·02	29·83	16·62
Shukers *et al.* (1938)	40·0	3·6	9·0	49	31·8	..	..	..	..	..

TABLE II—*concld.*

Author.	MEAN CORPUSCULAR VOLUME. In cubic microns (cμ).					MEAN CORPUSCULAR HÆMOGLOBIN CONCENTRATION PERCENTAGE. In percentage.				
	Mean.	Standard deviation.	Co-efficient of variation.	Maximum.	Minimum.	Mean.	Standard deviation.	Co-efficient of variation.	Maximum.	Minimum.
Present authors (1940), group A.	**70·54**	6·75	9·58	85·77	46·84	**32·54**	4·38	13·5	42·93	25·67
Present authors (1940), group B.	**87·66**	11·25	12·83	107·31	67·20	**27·85**	1·87	6·714	38·82	22·54
Shukers *et al.* (1938) ..	..	..	..	..	..	..	..	..	..	..

BLOOD GROUPS OF COMMUNITIES IN CALCUTTA.

BY

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I. The figures and their source.

In Table I are given figures taken from the last ten years' records of the Blood Transfusion Service which has been run from this laboratory for the hospitals in Calcutta for the last fifteen years.

II. The technique of grouping blood.

The technique has of course improved with the passage of time. It has always possessed the following features : (*i*) preparations on slides, uncovered, for macroscopic reading, (*ii*) r. b. c. washed and made into 2 per cent (or about 2 per cent) of suspension (whole blood not used), (*iii*) serum separated from the clot and inactivated if necessary (plasma not used), (*iv*) equal volumes of r. b. c. suspension and serum used (a volume of saline not added), error if any on the side of excess of serum, (*v*) time allowed at least 15 minutes, and (*vi*) *both the red blood cells and the serum tested.*

Most of the subjects tested have been either donors or recipients of blood. Their bloods have been matched directly also, after grouping. Incidentally, the correct grouping of the great majority of the subjects has been confirmed biologically with successful transfusions.

Using this technique no 'defective' groups or anomalous reactions have been found in bloods of healthy subjects although a sharp look out has been kept for them for the last five years. Even in the bloods of diseased subjects incompatibility within the same group or other anomalous reactions due to morbid causes, have been encountered on rare occasions only.

Lately, the quantities of the r. b. c. suspension (2 per cent) and the serum have been standardized. One drop of each from a capillary pipette delivering 50 drops to a c.c. has been used. The reaction has been done at room temperature in a

moist chamber made with a Petri-dish containing a wet piece of filter-paper in a watch-glass. Observation has been extended to 30 minutes. The preparations so made and kept do not dry up for 24 hours. Further, the agglutinating sera a and b have been selected. Their titre, *for a reaction appearing within a minute,* has not been less than 1 in 5.

III. SUPERIORITY OF FIGURES DERIVED FROM ROUTINE WORK OVER THOSE OBTAINED BY SPECIAL EFFORTS.

It is suggested that, with the development of blood transfusion service centres, figures of this kind preserved by competent serologists will, in the course of a few years, supply more useful and reliable information concerning the distribution of blood groups in India than has been collected by field workers. The average field worker, in his zeal speedily to leave his footprints on the sands of anthropology or genetics, more often than not employs rough and ready methods. Fingers are pricked at weekly markets and fairs, more often than not without consideration for asepsis ; whole blood of the subject is used ; testing sera are not selected ; and preparations are not made and examined with any attempt at uniformity. Speed rather than accuracy dominates the effort.

It will pay the field worker to make contact with and look upon a fully equipped serological laboratory as his headquarters as much as the library and the writing desk. There are pitfalls in the technique of iso-hæmagglutination. Their acquaintance must be made. Field work need not be needlessly inaccurate.

IV. STATISTICAL EXAMINATION OF TABLE I.

For this examination the writers are indebted to the Department of Vital Statistics and Epidemiology, All-India Institute of Hygiene & Public Health, Calcutta.

1. p, q, and r fulfil the requirements of the formula $p + q + r = 1$.

2. The difference between the British soldiers stationed in Calcutta and the Europeans of Calcutta is significant. The difference is primarily due to the significantly larger proportion of Europeans with B blood group.

3. The difference between the I. M. D. students and other members of the same Anglo-Indian community is not significant.

4. The difference between the Calcutta Europeans (consisting of a very small military population and a very large non-military population) and the Anglo-Indians is not significant.

5. The difference between the Calcutta Europeans (and Anglo-Indians) and the English and the Germans of Munich of Table II is significant. The significance is mainly due to the larger proportion of Calcutta Europeans with blood group B and the smaller proportion of them with blood group A.

6. The difference between the Calcutta Europeans (and Anglo-Indians) and the Germans of Berlin and Vienna is significant. The significantly larger proportion of the Calcutta Europeans with blood group O is responsible for the difference.

7. The difference between the Calcutta Europeans (and Anglo-Indians) and English in Australia of Table II is significant, the significantly larger proportion with blood group B and smaller proportion with blood group O of the Calcutta Europeans being responsible for the difference.

8. The difference between Brahmins, other high caste Bengalees and depressed class Bengalees in Calcutta is not significant.

9. The difference between the Calcutta Hindus and the U. P. Hindus (all castes, Table II) is significant. The significantly larger proportion among Calcutta Hindus with blood group O is mainly responsible for the difference.

10. The difference between the Hindus and the Mohammedans in Calcutta is not significant.

The frequency of general distribution has been worked out in each case for satisfying that the population is homogeneous. For establishing the resemblances and differences in the various population groups the significance tests have been carried out on the phenotype frequencies which are the original observations.

The analysis was carried out by means of χ^2-technique.

V. Observations on Table I.

(*i*) *The European figures for Calcutta are quite unlike the European figures from elsewhere.*—They approximate to the Anglo-Indian figures. Inclusion of the Anglo-Indians with the domiciled Europeans and of the latter with the European mercantile community resident in Calcutta is likely. The Presidency General Hospital, Calcutta, from the in-patient department of which the material was obtained, is essentially a hospital for the European population of Calcutta. A few non-European patients are also admitted. They have been excluded from the table. Jews and Armenians (living like Europeans) have also been excluded.

(*ii*) *The Anglo-Indian figures show a distinct rise in B but remain of the European type inasmuch as A is appreciably higher than B.*—All the subjects tested have, on the whole, approximated to the European type in stature, build, features, and complexion. In this respect a selection in favour of the type has operated.

So far as the writers are aware no other figures of this community are available.

(*iii*) *The figures for Vaidyas (also spelt Baidyas) though derived from a small number are striking.*—O predominates for no obvious reason. This community though not large is widespread. Inbreeding is excluded by the general Hindu laws of marriage. On the contrary, according to Risley (1891), Porter (1933), and common belief, the origin of this caste of the physicians of Bengal is more mixed than that of the other high Hindu castes. The writers have found (unpublished work) such predominance of O, in this region of Asia, only in the Gurkha and the Burmese troops.

(*iv*) *The Hindu figures differ from those of Malone and Lahiri (1929) for U. P. Hindus.*—The difference lies in an increase in O. This is contrary to the expectations based on Malone and Lahiri's conclusion to the effect that O increases as one travels up the Ganges valley.

TABLE I.

Blood groups of 2,472 subjects of Calcutta population.

Communities.	Total subjects.	O Total.	O Per cent.	A Total.	A Per cent.	B Total.	B Per cent.	AB Total.	AB Per cent.	p	q	r	p + q + r = 1·0
Europeans :—													
Loyal Regt. and K. R. R. C.	48	24	50·0	21	43·75	2	4·16	1	2·08	0·264	0·032	0·707	1·003
Others (mercantile community and domiciled Europeans. May include Anglo-Indians).	440	189	42·9	143	32·5	84	19·09	24	5·45	0·213	0·132	0·656	1·001
Total Europeans ..	488	213	43·6	164	33·6	86	17·6	25	5·12	0·218	0·121	0·660	0·999
Anglo-Indians :—													
I. M. D. students ..	67	27	40·29	28	41·79	10	14·92	2	2·98	0·257	0·094	0·632	0·983
Others (European ancestry may be remoter).	279	102	36·5	103	36·9	57	20·4	17	6·09	0·245	0·143	0·604	0·992
Total Anglo-Indians ..	346	129	37·2	131	37·8	67	19·3	19	5·4	0·248	0·134	0·603	0·990

Indians :—													
(i) Hindus :—													
Brahmins ..	201	76	37·8	40	19·9	71	35·3	14	6·9	0·145	0·240	0·615	1·000
Vaidyas ..	50	32	64·0	8	16·0	9	18·0	1	2·0	0·094	0·106	0·8	1·000
Kayasthas ..	149	53	35·5	29	19·4	60	40·2	7	4·6	0·128	0·258	0·596	0·982
Other high caste Bengalees (exact caste not determinable from records. Includes Vaisyas in addition to the above three castes).	504	165	32·7	109	21·6	182	36·1	48	9·5	0·169	0·263	0·574	1·006
Depressed class Bengalees (scheduled and non-scheduled).	160	62	38·7	38	23·7	49	30·6	11	6·8	0·168	0·210	0·624	1·002
Non-Bengalees ..	238	81	35·3	60	25·2	80	33·6	17	7·1	0·169	0·223	0·594	0·986
Total Hindus ..	1,302	469	36·02	284	21·8	451	34·6	98	7·5	0·160	0·238	0·6	0·998
(ii) Mohammedans ..	321	95	29·5	79	24·6	117	36·4	30	9·3	0·188	0·265	0·543	0·996
(iii) Other communities (Parsis, Jews, and Indian Christians).	15	7	46·6	5	33·3	2	13·3	1	6·66	0·225	0·106	0·683	1·014
Total Indians ..	1,638	571	35·03	368	22·5	570	34·9	129	7·9	0·163	0·242	0·592	0·997

Total Calcutta population tested .. 2,472 (Indians 1,638. Non-Indians 834).

TABLE II.

Figures for comparison from previous work.

Population.	Author.	Number tested.	PERCENTAGE OF GROUPS.				GENES.			
			O	A	B	AB	p	q	r	$= 1.0$
English, London ..	Kirwan and Taylor *	500	40·4	46·8	9·6	3·2	0·293	0·060	0·636	0·989
" " ..	Taylor and Ikin (1939)	1,073	45·9	42·0	9·3	2·7	0·257	0·062	0·677	0·996
" (troops) ..	Hirszfeld and Hirszfeld †	500	46·4	43·4	7·2	3·0	0·268	0·052	0·681	1·001
" Australia ..	Tebbut *	1,176	52·6	36·8	7·4	3·0	0·225	0·055	0·726	1·001
British troops, Kasauli ..	Malone and Lahiri (1929)	147	..	..	..	..	0·317	0·032	0·652	1·001
Germans, Berlin ..	Schiff *	5,621	36·1	42·6	14·9	6·4	0·286	0·113	0·601	1·0
" Munich ..	Kiuse *	1,300	42·5	43·0	9·0	4·9	0·269	0·067	0·653	0·989
" Vienna ..	Corvin *	6,934	34·4	45·2	15·0	5·4	0·301	0·112	0·587	1·0
Hindus, various castes, from U.P.	Malone and Lahiri (1929)	2,357	30·2	24·5	37·2	8·1	0·179	0·261	0·549	0·989
Indians, Calcutta ..	Greval, Chandra and Woodhead (1939).	300	26·7	26·7	37·7	9·0	0·197	0·269	0·516	0·982

* Taken from Weiner (1935). † Taken from Snyder (1929).
$p + q + r$ changed to unity from 100 of some records.

The difference between the Brahmins at one end of the social scale and the depressed classes at the other is not significant. The writers, therefore, conclude that looking for minute differences, in small collections of figures, from odd places and professions in Bengal, is not likely to prove profitable.

(*v*) *The difference between the figures of the various Indian communities, Hindus and Mohammedans, with the exception of the Vaidyas and 'other castes' (a very small group) is not significant.*—The figures for Mohammedans in the writers' collection are more representative of the general population than they would be when collected from small localities where inbreeding occurs. This inbreeding, incidentally, is the essential difference between small and *isolated* Hindu and non-Hindu populations. Even when it occurs amongst the Hindus, in Southern India and parts of the Bombay Presidency, it is much more restricted than it is amongst the non-Hindus.

The lack of significant differences between the various Indian communities in Calcutta further supports the conclusion that search for differences in small Bengalee communities, Hindu or Mohammedan, is not likely to prove profitable.

VI. Certain Amendments and Elucidations Concerning Figures Usually or Likely to be Quoted.

(*i*) *English (Liverpool) figures of Jones and Glynn.*—These figures reputed to be from 1,600 subjects have appeared in a book and are likely to be quoted by workers. Taylor and Prior (1938) have shown that the figures have been confused with 1,600 tests on 40 subjects only.

(*ii*) *Northern India figures of Malone and Lahiri* (loc. cit.).—They have also appeared in a book. In the original paper they are stated to be derived from the Hindus of various castes in the United Provinces of Agra & Oudh. They are figures for U. P. Hindus only, as has been stated in the table for comparison.

(*iii*) *Hindoos of the American books.*—The term Hindoo or Hindu of course includes all natives of India.

(*iv*) *Calcutta Indian figures of Greval, Chandra and Woodhead* (1939) *given in the table for comparison.*—The Indian figures were derived from 300 subjects. In them B was higher than A as expected. A and O, however, were equal. This work was undertaken for the determination of the types M, N, and MN. As typical of the distribution of the groups in Calcutta Indians, the present figures, which are derived from a much larger number, are to be preferred.

The figures of this small series are excluded from those in Table I which, as stated before, are taken from the register of donors and recipients of blood. The material for the small series was obtained from specimens received in this laboratory for Wassermann test. Fresh clots were washed for red blood cells which alone were tested.

Summary.

1. From the records of the blood transfusion service run by the department of the Imperial Serologist, Calcutta, a table giving the blood groups of 2,472

subjects of the Calcutta population, by communities, is compiled. Another table is compiled from previous workers' figures for comparison.

2. Essential features of the technique used in grouping subjects are given.

3. It is pointed out that figures collected in the routine of a serological laboratory are superior to those of a field worker who more often than not employs rough and ready methods.

4. Statistical examination of the figures is given.

5. The following points emerge: (*i*) figures for the Calcutta European community, 488 subjects, are unlike those of other European communities; (*ii*) figures for the Calcutta Anglo-Indian community, 346 subjects, are like those of the Calcutta European community; (*iii*) figures for the Calcutta Hindus, 1,302 subjects, with the exception of the Vaidyas, are alike, regardless of the social scale and contrary to expectation differ from those of U. P. Hindus in an increase in O ; (*iv*) figures for the Vaidyas, 50 subjects, show a remarkable predominance of O ; (*v*) figures for the Calcutta Mohammedans, 321 subjects, are like those for the Calcutta Hindus ; and (*vi*) looking for minute differences in small and isolated communities in Bengal, Hindu or Mohammedan, is not likely to prove profitable.

6. Certain incomplete and erroneous references and descriptions are amended and elucidated. They concern : (*i*) Jones and Glynn's figures for the Liverpool English, (*ii*) Malone and Lahiri's figures, in a book, for Northern India, (*iii*) Hindoos of the American authors, and (*iv*) Greval, Chandra and Woodhead's figures for Calcutta Indians.

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Postscript.—

The following passage from a letter which appeared on page 4 of *The Statesman*, Monday, the 15th January, 1940, may explain the similarity between the blood groups of the non-military European community and the Anglo-Indian community in Calcutta :—

"In any case it will be admitted that the number of domiciled European families is very small indeed. The families are constantly moving in Anglo-Indian circles and frequently intermarry with those of mixed descent.

     *   *   *   *   *   *

Calcutta, January 9.   Henry Gidney,
                                   Lieut.-Colonel, I.M.S. (*retd.*)."

RADIOLOGICAL STUDIES OF THE ALIMENTARY TRACT OF NORMAL MONKEY (*MACACUS SINICUS*).

BY

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(*An Inquiry under the Indian Research Fund Association.*)

(*From the King Institute of Preventive Medicine, Guindy, Madras.*)

DISORDERS of the alimentary tract are the result of dysfunction of an extremely complicated system finely adjusted and dependent upon various factors, chemical, biochemical, and neurological. Radiological studies of the alimentary tract in man, during recent years, have shown the extreme variability and adaptability of various parts of the tract to postural, physiological, and emotional responses. It would appear, therefore, that full cognizance must be taken of all these factors when experimental studies on this system are made and, in addition, the animal for experimentation should be one with an alimentary system closely resembling that of man.

A large amount of experimental work on this system has been carried out in various countries in connection with disorders pertaining to the digestive tract. Most of the workers have confined their experimentation to laboratory animals, such as the rabbit, dog, and rat, presumably due to the ease with which these animals can be obtained in most countries. In India, the monkey is very easily procurable and was considered a suitable animal to employ.

The author has, therefore, used it exclusively for feeding experiments in connection with experimental production of gastro-duodenal ulcers. Radiological study in man has shown how mere anatomical considerations are misleading. The living alimentary tract of normal monkeys, with the help of an opaque meal examination, has therefore been studied. The results of these observations are recorded in this short note.

The barium meal was made up in the usual manner as prepared for human cases. A preparation of Horlicks and barium sulphate sold under the proprietary name 'Horlicks Shadow Food' was used and found quite suitable. The meal did not seem to upset the animals in any way.

(1117)

The animal was tied to a wooden cross by means of bandages. In order to prevent the animal from moving about much and making it difficult to take X-ray exposures, it was found convenient to fix the hips and hind limbs with tight bandages. The abdomen and thorax were lightly bandaged to prevent distortion of the shape of viscera in the abdomen and limitation of the respiratory movements of the thorax. The fore-limbs were also tied. Animals so tied behaved perfectly and could be screened and X-rayed with the greatest of ease in both the erect and recumbent positions.

The most convenient method of introducing the meal was by lightly anæsthetizing the animal after tying him on the cross and then introducing No. 12 rubber catheter into the œsophagus and injecting the meal with a 20-c.c. syringe. Forty c.c. of the barium meal was found to be the average quantity sufficient to fill two-thirds of an empty stomach. All animals were starved after the evening meal of the day previous to examination.

After the introduction of the barium meal, repeated screen examinations were carried out at varying intervals to note the movements of the meal. Records were made at definite intervals and X-ray photographs taken. The animals were kept in the erect position during the examinations and were given nothing to eat or drink until after the six hours' examination. Anæsthesia was only required for the introduction of the meal.

The first experiment revealed the profound influence the surroundings have on the movement of the alimentary tract of the monkey. The introduction of the meal under chloroform, the low temperature of the air-conditioned rooms of the Barnard Institute, and disturbing conditions in the X-ray rooms owing to lights being switched on and off coupled with the fact of the animal being tied down to the wooden cross resulted in almost complete inhibition of the intestinal movements. In this experiment, the stomach of the animal remained full for several hours until the animal was released and returned to the cage. It may be noted here that in man the psychological factor causes excitation and rapid exit rather than retention as was observed in the monkey.

It was deemed necessary, therefore, to release the animal after each examination, to reduce the number of examinations and to keep the animal in the X-ray room all the time to get him used to the surroundings and the temperature. With these modifications in procedure, repeatable results were obtained.

Plates XLIII to XLVII show the barium meal pictures of a monkey. It will be seen that in this animal, under the conditions of the experiment, the stomach appears to be of the 'J' pattern rather than the 'L' or the 'steer horn' patterns classified in man.

The peristalsis is observed to originate from where the rather long fundus joins the body of the stomach and it becomes deeper as it approaches the pyloric area. The pyloric canal is well formed. Screen examination and stimulation of the stomach wall by palpation induces peristaltic activity. The opaque meal begins to enter the duodenum immediately after it is introduced into the stomach. The shape of the duodenum gets elongated and it appears to retain the meal until

PLATE XLIII.

Barium meal examination on monkey.
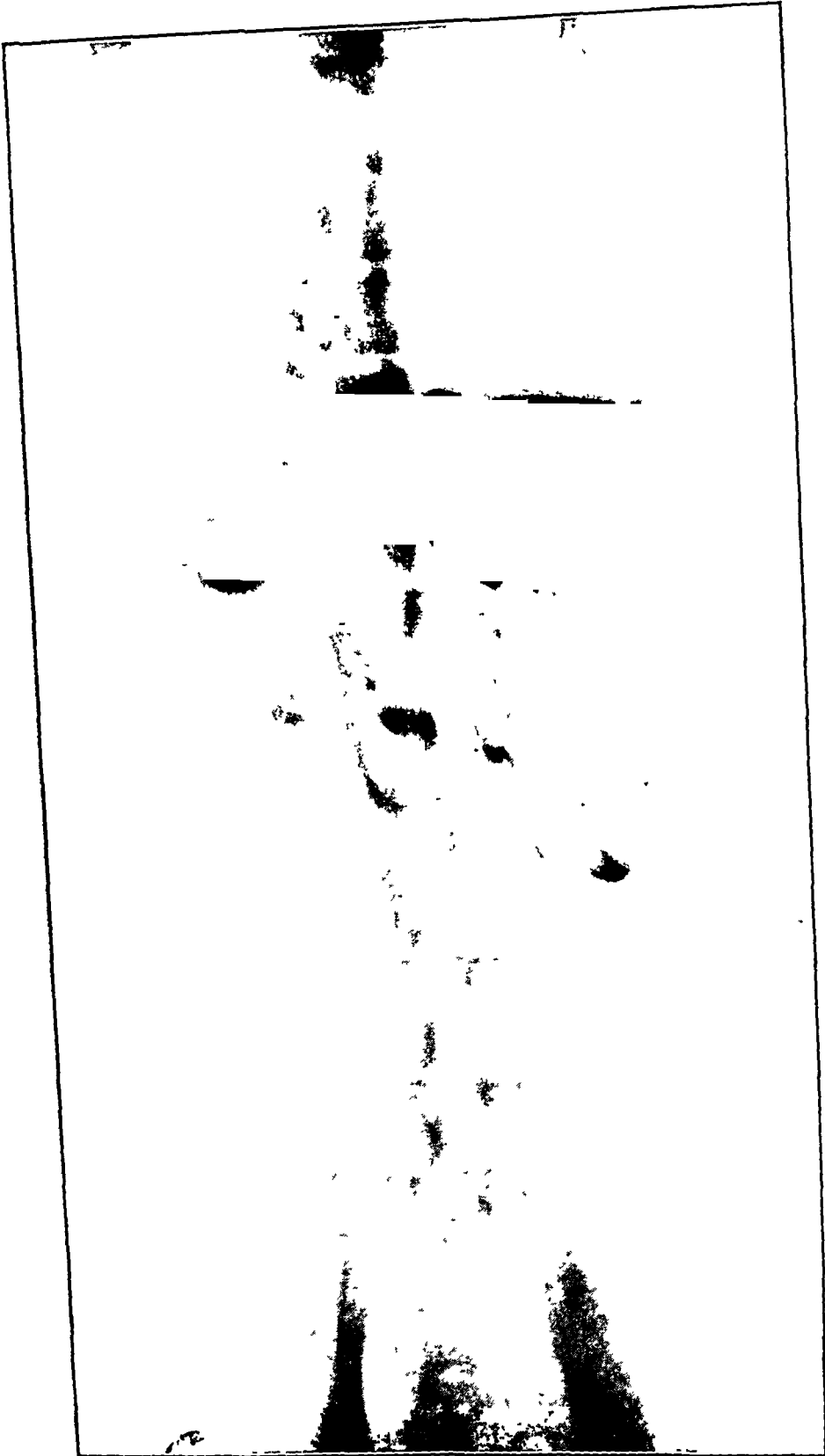
Taken immediately after the introduction of the barium meal—The outline
of the stomach in the erect position is shown with formation of the duodenal
cap. Portion of the meal has already escaped into the jejunum.

PLATE XLIV.

Barium meal examination on monkey.
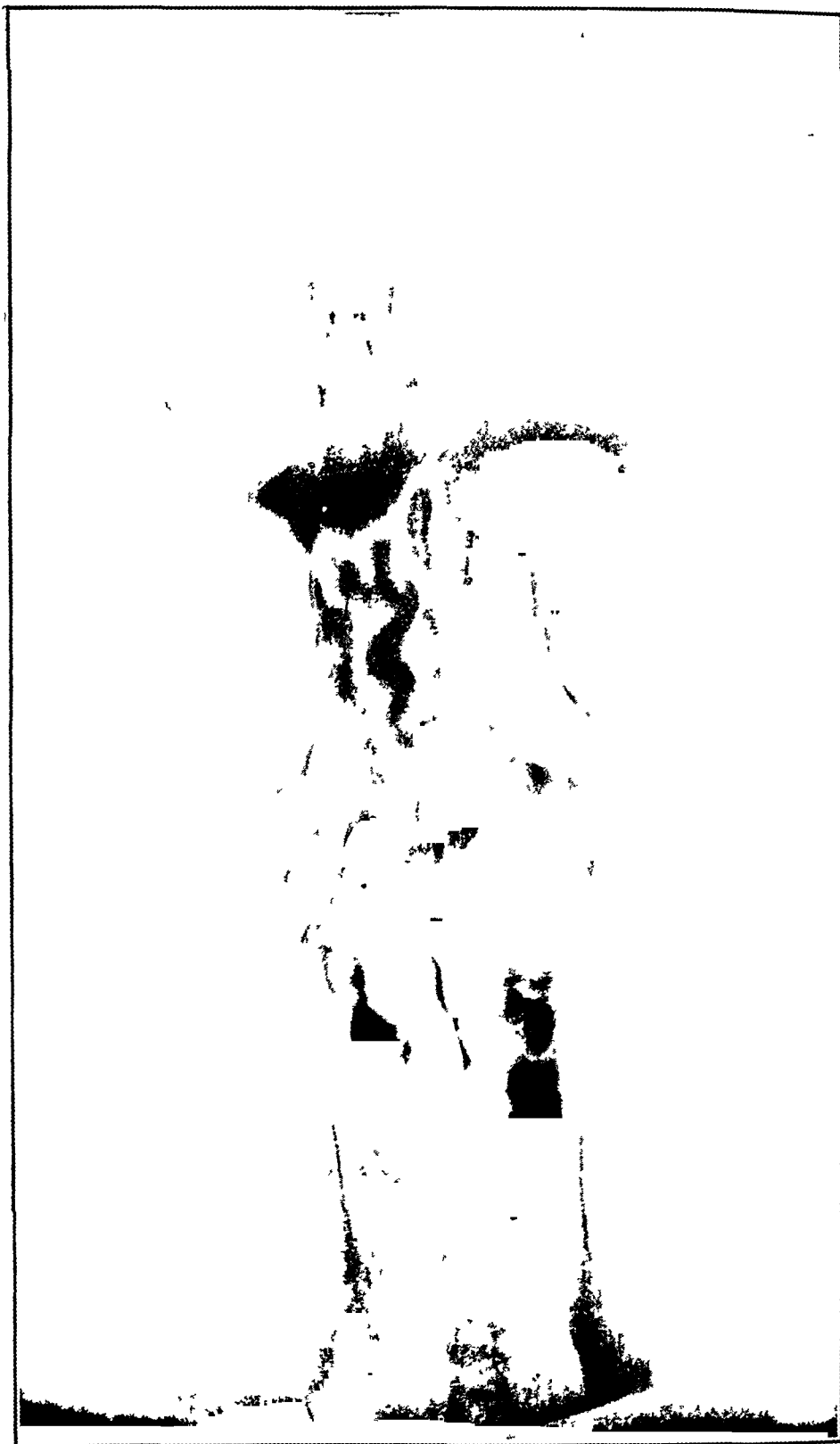
Two hours after the meal—The stomach is emptying. The first part of the duodenum is seen full. A large portion of the meal is seen in the small intestine.

PLATE XLV.

Barium meal examination on monkey.
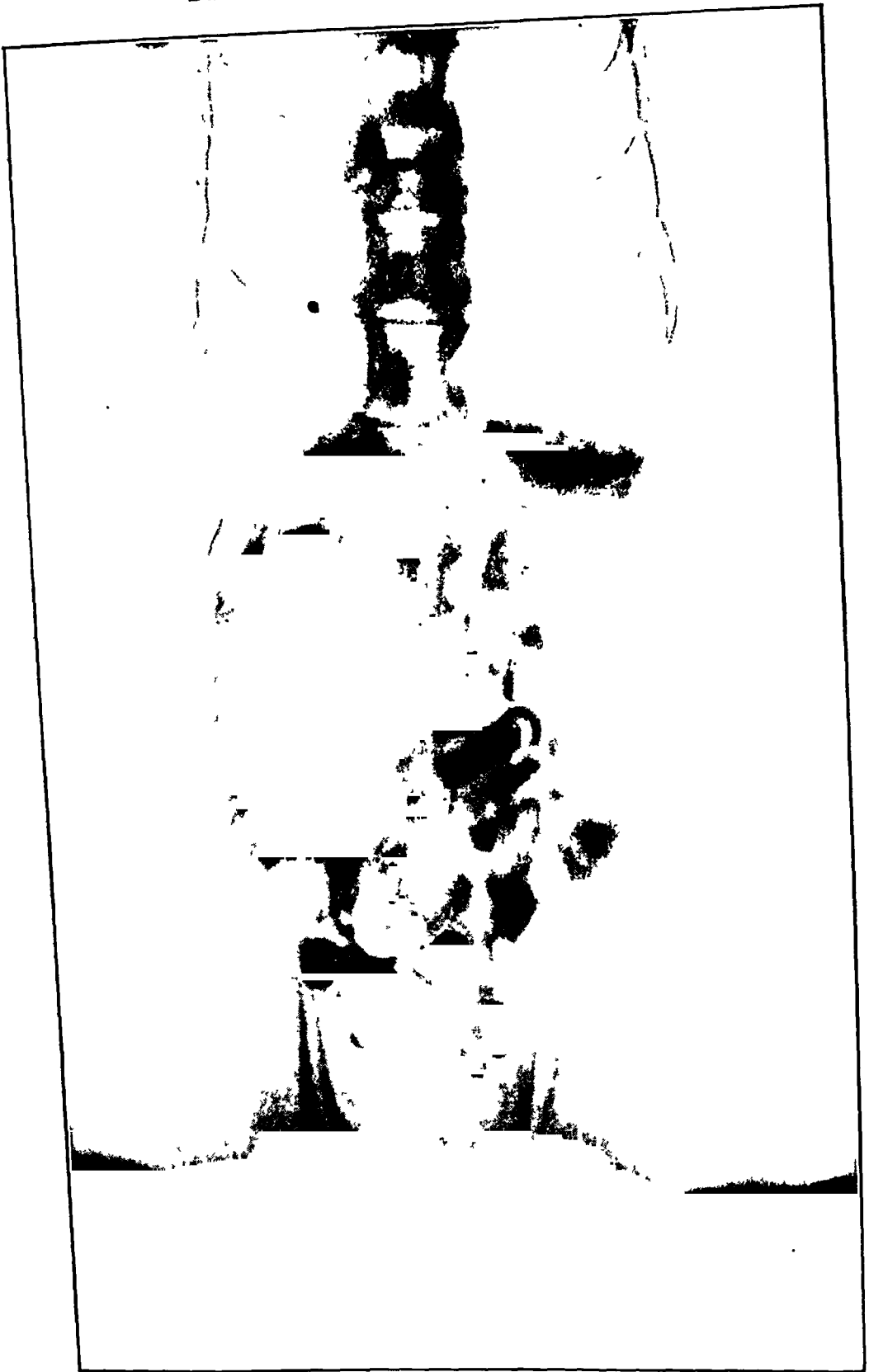
Three-and-a-half hours after the meal—The stomach is nearly empty. The first part of the duodenum is seen full. Meal is seen in the cæcum and small intestine.

PLATE XLVI.

Barium meal examination on monkey.

Five-and-a-half hours after the meal—The stomach is empty. The cæcum is completely filled. Portion of the meal is still in the small intestine.

PLATE XLVII.

Barium meal examination on monkey.
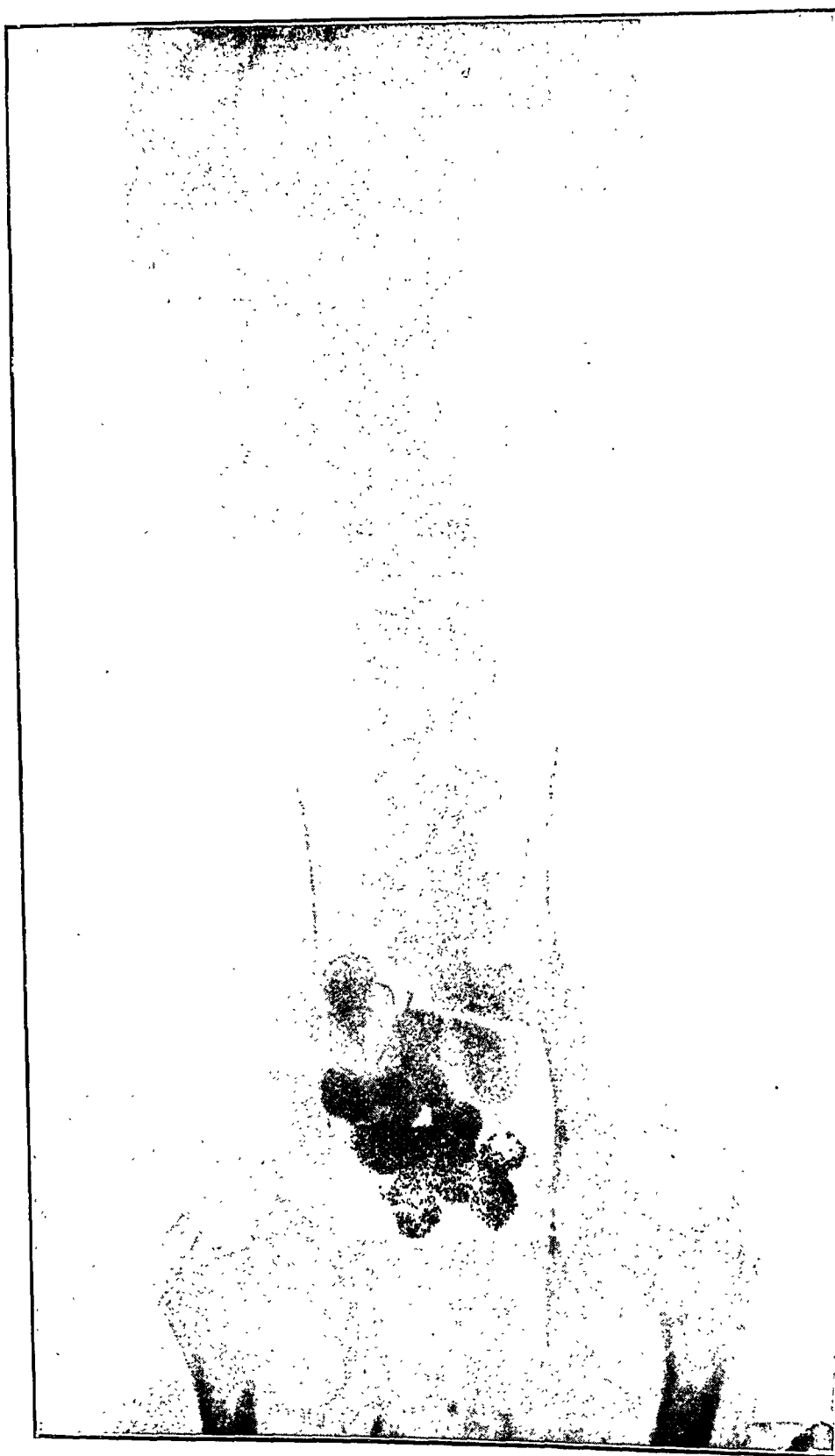
Twenty-four hours after the meal—No meal is left in the stomach, duodenum, small intestine, or the cæcum. It is now seen in the descending colon, pelvic colon, and the rectum.

PLATE XLVIII.

Barium meal examination on monkey.
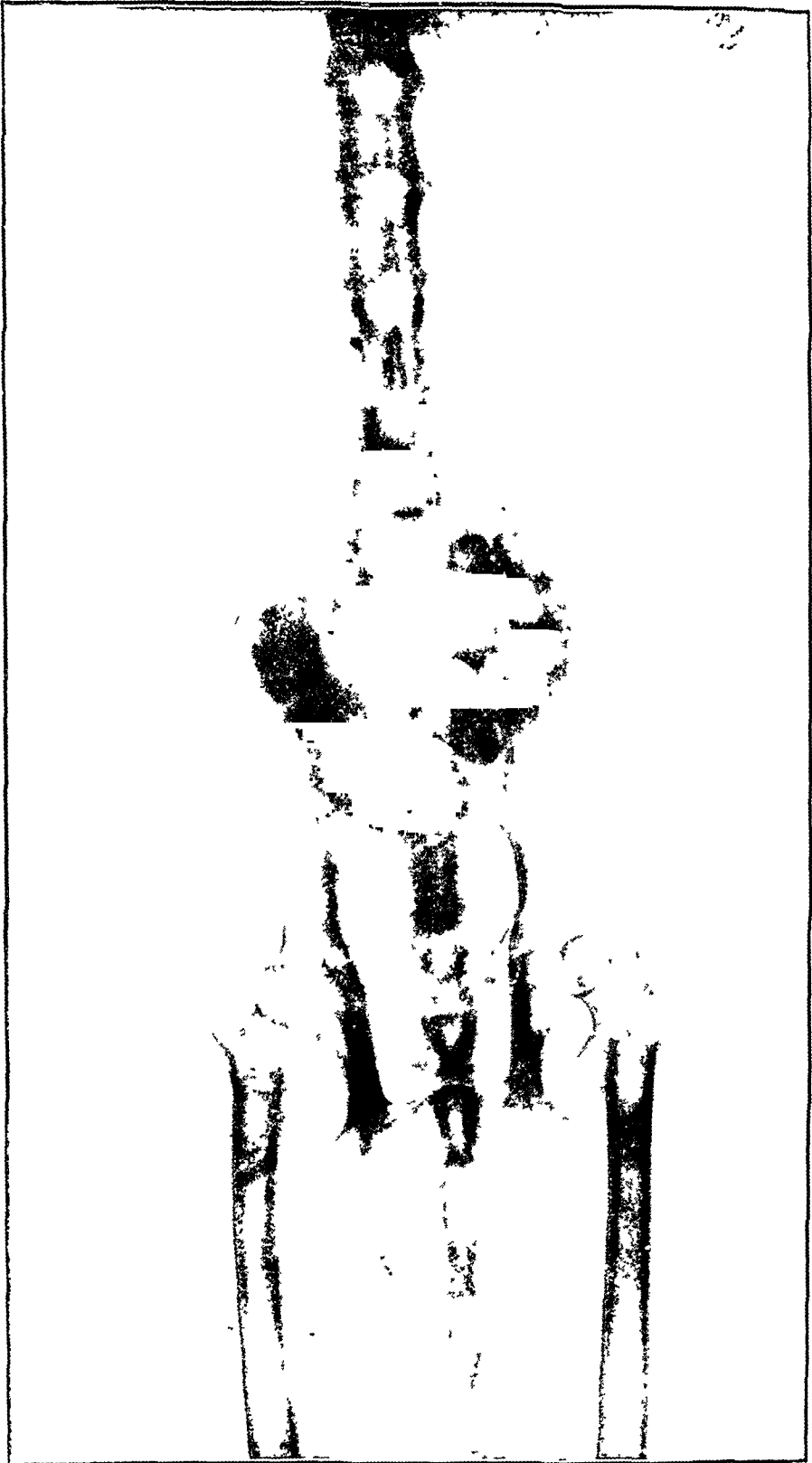
Twenty-four hours after the meal—The well-marked rectal ampulla is well seen.

it gets fully filled when it empties itself into the lower portions of the duodenum. The body of the stomach and the pyloric antrum merge into each other before the contents are passed into the first part of the duodenum. The duodenal filling and emptying co-ordinate with the contractions and dilatations of the pyloric antrum. The average emptying time for the monkey stomach was $3\frac{1}{2}$ to 4 hours (five experiments).

The small intestine shows the usual movements seen in man except that the movement of the terminal coils of the ileum is of much greater amplitude than in man. When it is filled, the terminal coils of the small intestines are situated lower down in the pelvis.

The cæcum persistently remains just above the crest of the ileum. It is completely filled with the barium meal in about six hours.

The entire meal escapes into the descending colon and rectum in 24 hours. The haustrations of the descending colon are well marked and the rectal ampulla is much more definitely shown in the monkey than in the human subject (Plate XLVIII).

The anatomical considerations, which are not detailed here, and the radiological studies recorded, sufficiently indicate the close resemblance of the alimentary tract of man and monkey.

SUMMARY.

Radiological studies of the alimentary tract of *Macacus sinicus* after an opaque meal are recorded and the close resemblance of the physiological reactions of the alimentary tract of man and monkey shown.

ACKNOWLEDGMENTS.

THE REACTION BETWEEN *VIPERA RUSSELLII* VENOM AND ITS ANTIVENENE.

BY

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AND

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IT has been shown in two papers published by one of us (Ghosh, 1935, 1936) that the combination of antigens with their respective antibodies can be accounted for quantitatively by equations based on the theory of surface reaction or adsorption. Of the various types of combination which are theoretically possible, only three have so far been considered in detail and they are as follows :—

1. An antigen particle occupies one active point on the surface of an antibody particle.

2. An antigen particle occupies two active points on the surface of an antibody particle.

3. An antigen is a mixture of two types of particles having properties envisaged under (1) and (2).

The mode of combination of Russell's viper venom with its antiserum was investigated with a view to ascertain if it can be included under any of the three types enumerated above. The results which we have obtained indicate that the reaction is of the type (1) and the equation representing the number of units of venom (one unit of venom = one pigeon m.l.d. = 0·01 mg.) neutralized by the antivenene has the form—

$$P = \frac{(K + C + TN) - \sqrt{(K + C + TN)^2 - 4TNC}}{2} \quad \ldots \ldots \quad (1).$$

In the above equation P represents the number of units of venom neutralized, C the number of units of venom taken initially, T the amount of antivenene added in c.c. and K and N are constants. N stands for the maximum number of units of venom which can be neutralized by 1 c.c. of a given sample of antivenene and is a characteristic constant of the sample.

It may be noted that the number of units of venom which remains free is (C—P) and it can be calculated with the help of the equation (1)—

$$(C - P) = C - \frac{(K + C + TN) - \sqrt{(K + C + TN)^2 - 4TNC}}{2} \qquad \ldots \quad (2).$$

EXPERIMENTAL.

A 0·1 per cent solution of Russell's viper venom in 0·9 per cent sodium-chloride solution was prepared and 2 c.c. of this solution containing 200 units were placed in each of a series of tubes numbered 1, 2, 3, 4, and so on. The amount of antiserum which was added to tube No. 1 was 0·1 c.c., to tube No. 2, 0·2 c.c., to tube No. 3, 0·3 c.c., and so on. Requisite amount of normal saline was finally added to each of the tubes so that the volume of the solution in each was 3·0 c.c. The tubes were carefully stirred to ensure thorough mixing of their contents and were placed in a thermostat at 37°C. for 30 minutes. The number of units of venom free in each tube was determined in the following way : Let us consider tube No. 4 which contains 2 c.c. of venom solution, 0·4 c.c. of antiserum and 0·6 c.c. of normal saline. Part of its contents was taken in a micro-syringe and injected intravenously in increasing doses into a number of pigeons of standard weight. Suppose the pigeons which received 0·37 c.c. and smaller doses survived and those which received 0·38 c.c. and higher doses died within 15 minutes. Then 0·38 c.c. of the mixture must have contained at least one unit of venom in the free state. Therefore the amount of venom which remains uncombined in 3 c.c. of the mixture is 3/0·38, i.e., 7·9 units. Each of the above experiments was repeated twice and in a few cases thrice. The results are recorded in Tables I and II. The amount of

TABLE I.

N = 756·30.

K =   5·18.

Units of venom taken C.	Number of c.c. of antivenene added T.	UNITS OF VENOM NEUTRALIZED.	
		Observed.	Calculated from equation (1).
200	0·10	75·0	72·7
200	0·15	107·0	107·4
200	0·20	140·0	139·3
200	0·25	163·5	164·7
200	0·30	175·0	180·0
200	0·40	192·2	191·0
200	0·50	197·0	195·0

venom which was neutralized by the antivenene was determined by subtracting the number of units of venom found free from 200 which represented the total number of units of venom taken initially.

TABLE II.

N = 756·30.

K =   5·18.

Units of venom taken C.	Number of c.c. of antivenene added T.	UNITS OF VENOM FREE.	
		Observed.	Calculated from equation (1).
200	0·10	125·0	127·3
200	0·15	93·0	92·6
200	0·20	60·0	60·7
200	0·25	36·5	35·3
200	0·30	25·0	20·0
200	0·40	7·8	9·0

EFFECT OF DILUTION.

The effect of dilution of the solutions of venom and the antivenene on their mutual reaction was investigated. The venom was diluted so that 2 c.c. of its solution contained 100 units. The antiserum was also diluted, 1 c.c. being made up to 3 c.c. The rest of the experimental procedure was exactly the same as before. The final volume of the mixture was also 3 c.c. in each of the tubes. It follows from theoretical considerations that the equations (1) and (2) should be applicable to this case also; only the constant N which is characteristic of the antiserum should have for this diluted system one-third of its former value owing to the antiserum being diluted three times. The other constant K depends only on the temperature at which the reaction occurs and hence should not change with dilution. These theoretical conclusions are fully borne out by experimental facts. The results are recorded in Tables III and IV. It will be noticed that the agreement between the observed and calculated values is quite good.

TABLE III.

N = 252·10.

K =   5·18.

Units of venom taken C.	Number of c.c. of antivenene added T.	UNITS OF VENOM NEUTRALIZED.	
		Observed.	Calculated from equation (1).
100	0·1	25·0	23·6
100	0·2	46·0	46·0
100	0·3	66·3	65·7
100	0·4	82·4	80·1
100	0·5	88·3	88·1
100	0·6	91·9	92·0
100	0·7	93·4	94·1

TABLE IV.

N = 252·10.

K =   5·18.

Units of venom taken C.	Number of c.c. of antivenene added T.	UNITS OF VENOM FREE.	
		Observed.	Calculated from equation (2).
100	0·1	75·0	76·4
100	0·2	54·0	54·0
100	0·3	33·7	34·3
100	0·4	17·6	19·9
100	0·5	11·7	11·9
100	0·6	8·1	8·0
100	0·7	6·6	5·9

The Danysz phenomenon in the reaction between Vipera russellii *venom and its antitoxin.*

It has been reported (Arrhenius, 1907) that in the reaction between cobra poison and its antibody, Danysz phenomenon does not occur. The experiment has recently been repeated by us using cobra hæmolysin and our results are also similar to those of early workers. We next investigated the reaction between *Vipera russellii* venom and its antitoxin with a view to ascertain if it shows Danysz phenomenon. In a preliminary experiment the amount of antitoxin which, when added to a given amount of venom in one instalment, just neutralized it, was determined. Two series of tubes were then set up each containing the same amount of antitoxin and the given amount of venom was added to series I tubes in one instalment and to series II tubes in two instalments at an interval of 30 minutes. The mixtures were then incubated at 37°C. for thirty minutes and injected into pigeons weighing 290 g. to 300 g. The results are recorded in Table V in which the ' — ' sign indicates that the pigeons did not die within 3 hours and the ' + ' sign indicates that the pigeons died within 10 minutes of the injection. It will be noticed that the contents of tubes of series II in which the venom was added in two instalments were decidedly more toxic than those of series I.

TABLE V.

Series I.

Tube number :—	1	2	3	4	5	6
Volume of antitoxin added in c.c.	1	1	1	1	1	1
Volume of venom in c.c. added all at once.	1·4	1·4	1·4	1·4	1·4	1·4
Effect on pigeons ..	—	—	—	—	—	—

Series II.

Tube number :—	1	2	3	4	5	6
Antitoxin added in c.c. ..	1	1	1	1	1	1
Volume of venom in c.c. added per instalment at 30 minutes' interval.	0·7	0·7	0·7	0·7	0·7	0·7
Effect on pigeons ..	+	+	+	+	+	+

Flocculation test in mixtures of venom and antivenene.

The possibility of titration of cobra antivenene by precipitation reaction was first investigated by Lamb (1904). He could not find any relation between the potency determined by animal experiment with that determined by precipitation reaction. Calmette and Massol (1909), on the other hand, claimed that it was possible to determine the potency of antivenene by precipitation reaction. Recently, Mallick (1935) tried to determine the potency of the Kasauli antivenene against cobra venom and failed to confirm the results of Calmette and Massol. We also studied the flocculation reaction of antivenene against protein fractions separated from cobra venom and found that the flocculation reaction occurs with a protein fraction which does not contain the neurotoxin. Therefore, both Mallick's and our results are in agreement with the observation of Lamb that the precipitation reaction cannot be used for the *in vitro* titration of antivenene against cobra (*Naja naja*) venom. The possibility of titration *in vitro* of antivenene against *Vipera russellii* venom was also investigated by us. Of a series of tubes containing *Vipera russellii* venom and antivenene in different proportions, the one containing the balanced mixture was found to develop greater turbidity than the others. The results obtained from four different samples of antivenene tried so far, are recorded in Table VI. A unit of antivenene is taken to be that amount which can neutralize 1 m.l.d. of venom (tested on pigeon).

TABLE VI.

Number of antivenene sample.	POTENCY DETERMINED BY	
	Animal experiment.	Turbidity test.
	Units.	Units.
1	180	175
2	320	300
3	318	285
4	680	667

CONCLUSIONS.

1. It has been shown that the neutralization curve of *Vipera russellii* venom by its antivenene can be represented by an equation of the form—

$$P = \frac{(K + C + TN) - \sqrt{(K + C + TN)^2 - 4TNC}}{2}.$$

2. When a given quantity of *Vipera russellii* venom is added to an equivalent quantity of the antivenene in two instalments at an interval of 30 minutes, the resulting mixture develops slight toxicity.

3. Some observations have been recorded which indicate the possibility of titration *in vitro* of antivenene against *Vipera russellii* venom by comparison of turbidity developed in venom-antivenene mixtures.

ACKNOWLEDGMENT.

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ERROR IN THE ESTIMATION OF THE MOST PROBABLE NUMBER OF ORGANISMS BY THE DILUTION METHOD.

BY

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DILUTION method is designed for the estimation of the number of organisms present in a certain quantity of water. The method consists primarily in preparing several different dilutions of the original water sample. From each of these dilutions a number of samples of known quantity are drawn and each of these samples is inoculated in separate tubes in liquid media. The presence or absence of organisms in each tube is indicated by gas formation. The records thus obtained, i.e., of the number of tubes showing evidence of the presence of organisms and of those failing to do so, are made the basis of estimation of the most probable number of organisms present in the original samples.

In a former publication (Swaroop, 1938) formulæ were given for the determination of the most probable number of organisms and of the standard error of this number and tables were provided for facilitating the calculations of these estimates. If the dilution factor is a (usually taken as 10), the number of tubes used for the xth dilution s_x, and if σ_n denote the standard error of the most probable number n then it was stated that—

$$\frac{1}{\sigma_n^2} = S \left\{ \frac{s_x E_x}{a^{2x}} \right\} \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots(1).$$

when S denotes summation over all the dilutions and E_x stands for the value of $\dfrac{1}{e^{n/a^x} - 1}$ for which tables were provided. This formula provides a direct estimate of the standard error in the estimation of the probable number and the purpose of this paper is to study, in the light of the above formula, the effects of the

(1129)

various factors which influence the error of determination of the most probable number.

Obviously a direct method of studying the efficiency of the dilution method will be to take samples containing known numbers of organisms and to carry out a large series of the usual dilution tests. Thus will be determined, for any known concentration, different values of the probable numbers and if such experiments are carried out, possibly an infinite number of times, under identical conditions, reliable information can be obtained about the error involved in this determination. Thus, the effect of such controllable factors as the number of tubes, number of organisms in the original supply, and the dilutions chosen for the test, can be studied. In actual practice, however, the method will not only be extremely laborious but beset with difficulties as regards taking samples with known number of organisms and preserving constant conditions of the experiment. The same information can, on the other hand, be deduced, with a fair amount of reliability, from a careful study of the formula (1).

The error of estimation of the probable number is a very vague term and in order to make it susceptible to numerical treatment it must therefore be clearly defined. For the purpose of the present study it appears reasonable to calculate error by expressing the standard error of the probable number as a percentage of the probable number.

In terms of symbols—

$$\text{The error of determination} = \frac{\sigma_n}{n} \times 100 \quad \dots \dots \dots \dots \dots \dots \dots \dots \dots \dots (2).$$

We may thus study how it can be possible for the test to give us an error less than a certain percentage of the probable number. It may, for instance, be of interest to know how the test may be planned so that the standard error may be only 10 per cent of the estimated probable number. Obviously the smaller this percentage the better will be the accuracy of the estimation and vice versa. This definition of error appears to be the simplest with which the present study may be undertaken. It may, however, be remarked that if a different definition is adopted the treatment in the discussion which follows will have to be suitably modified.

The problem now reduces itself to the nature of variation exhibited by $\frac{\sigma_n}{n} \times 100$, which may be expressed by the relation—

$$\frac{\sigma_n}{n} \times 100 = \frac{100}{\sqrt[n]{S\left(\frac{s_x \, E_{n/a}{}^x}{a^{2x}}\right)}} \quad \dots \dots \dots \dots \dots \dots (3).$$

It is clear from the above expression that the error of estimation depends upon the number of tubes s_x, the dilution factor a, the number of dilutions x and the probable number n. In other words error is related to all the relevant variables but the nature of this relation is not simple. Attempts were made to develop

most general mathematical formulæ to describe the independent as well as joint effects of these factors but complicated algebraic expressions were obtained. It was therefore thought desirable to study how the error would vary in those cases which frequently arise in practice. These cases have been studied in some detail.

CASE 1.—When the dilution factor is 10, three dilutions, $\frac{1}{10}$, $\frac{1}{10^2}$, and $\frac{1}{10^3}$, are taken and equal numbers of tubes are selected for each dilution.

In this case $a = 10$; $x = 1, 2$ and 3, and $s_1 = s_2 = s_3 = s$ (say).

As a preliminary step the values of—

$$\frac{100}{n\sqrt{S\dfrac{E_{n/a^x}}{10^{2x}}}} = \frac{100}{n\sqrt{K_1}} \quad (\text{say})\dots\dots\dots\dots\dots\dots\dots\dots\dots(4).$$

were tabulated for selected values of n. This expression differs from (3) in that the symbol s for the number of tubes is left out. The values of (4) are given in the third and the sixth columns of Table I which also shows the value of $\frac{1}{\sqrt{K_1}}$. It may be remarked that $\frac{1}{\sqrt{K_1}}$ measures the standard error to be attached to the estimated probable number when estimation has been carried out by taking one tube of each dilution.

Columns (3) and (6) of Table I set out corresponding to different values of probable number, the error arising when only one tube of each dilution has been taken. These figures show that although the function tabulated exhibits variations, these are, from a practical point of view, not of very material importance. The percentages being, except for very small values of n, all less than 150.

In order to study the gain in accuracy by taking larger numbers of tubes the values of the error of estimation of the probable number (n) have been calculated for a few selected pairs of values of the probable number (n) and the number of tubes (s) and these are set out in Table II.

Table II reveals that for the same number of tubes the error is, for practical purposes, of about the same order of magnitude over a very large range of values of the probable number. With an increase in the probable number of organisms the error of estimation does not show a tendency either of systematic increase or decrease. An increase in the number of tubes, however, produces a marked reduction in the error. Whereas, with one tube the error on the average may be of the order of 130, it drops to about 60 or less with five tubes, to 30 or less with 20 tubes, and to less than 10 when the number of tube is as high as 200. The Graph has been prepared for a detailed study of only a few values of n. Each curve drawn relates to a different value of n. It may be seen that the curve for n = 500 lies midway between those for n = 1 and n = 20. The error of estimation as already stated possesses no systematic variation with an increase in the

value of n. The curves show that a marked fall occurs in the error of estimation with a rise in the number of tubes in the beginning but for much larger number of tubes the drop in error is, for practical purposes, not so important.

<div align="center">GRAPH.</div>

Showing the reduction in error of estimation due to an increase in the number of tubes for three selected values of the probable number N.

N = 1 .... _____
N = 500 ___ _____
N = 20 ..__ x—x—
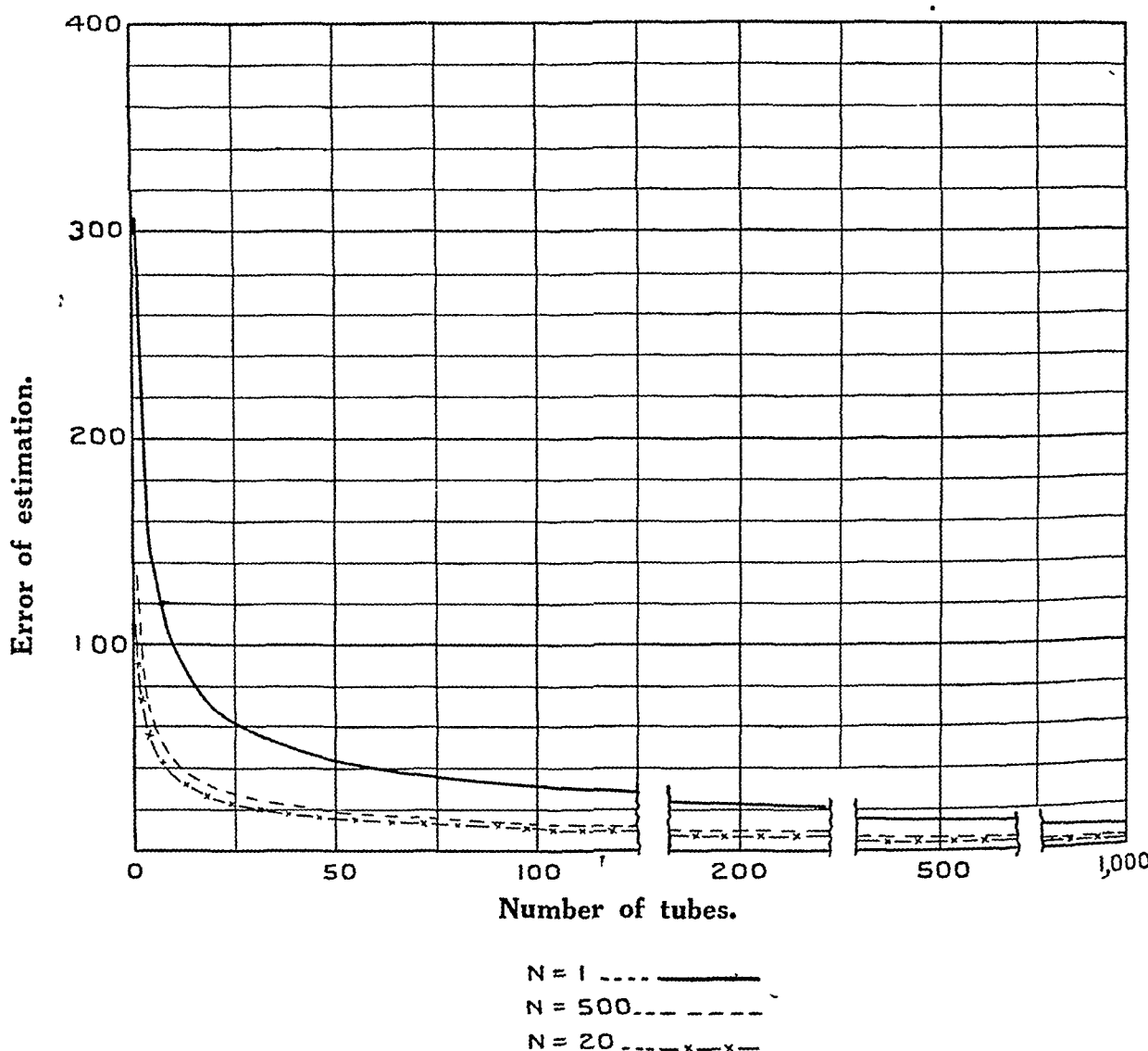
As mentioned previously, in some cases we may so like to arrange our test that an error larger than the one stipulated may not arise. For instance, before

starting the actual test we may decide on allowing for only 10 per cent error in our estimate. In a case like this it may be asked : how many tubes will it be necessary to inoculate against each dilution ? From an inspection of the formula (3) it may appear that without a knowledge of the probable number no answer may be possible. The answer does, to some extent, depend on the probable number itself but in the absence of its knowledge the experiment may be usefully planned as is shown below :—

$$\text{Error} = \frac{100 \times \sigma_{(n,\ 1)}}{n\sqrt{s}}$$

where $\sigma_{(n,\ 1)}$ denotes the standard error of n when only one tube is used for each dilution. It is clear from Table I that for the present purpose $\dfrac{100\ \sigma_{(n,\ 1)}}{n} = \dfrac{100}{n\sqrt{K_1}}$

may be considered to have the value of approximately 130. It is the approximate constancy of these values of $\sigma_{(n,\ 1)}$ which enables us to estimate s.

The relationship (2) then reduces itself to $\dfrac{130}{\sqrt{s}} = 10$ (approximately) or $s = 169$.

In other words we should start by taking about 170 tubes for each dilution. A more accurate result will be obtained by getting from preliminary trials a rough idea of the probable number n, and then base the calculation on $\dfrac{1}{n\sqrt{K_1}}$ derived from values of n lying near this figure.

CASE 2.—When the dilutions taken are 1/2, 1/10, and 1/100, and an equal number of tubes are taken for each dilution.

For this case the values of the function—

$$\frac{1}{\sqrt{\dfrac{E_{n/2}}{4} + \dfrac{E_{n/10}}{100} + \dfrac{E_{n/100}}{10000}}} = \frac{1}{\sqrt{K_2}} \quad (\text{say})\dots\dots\dots\dots(5).$$

have been calculated for several values of n and are presented in Table III. This function measures as previously stated the standard error of n, when one tube is taken for each dilution. The values of error of estimation, viz., $\dfrac{100\sigma_n}{n}$ in each case are also shown in the third and the sixth columns of Table III.

A comparison of these values of error of estimation for different values of n shows that by taking a different set of dilutions the error has comparatively increased for large values of n and decreased for small values.

The values of the ' error of estimation ' for a few selected pairs of values of n and s are presented in Table IV. It may be seen, on comparison with Table II, that the values of the ' error of estimation ' set out in this table are, especially for small values of n, less than those observed in case 1. This suggests that maximum

advantage gained from a chosen set of dilutions will depend on the concentration of organisms in the supply. This point is discussed in a later section in this paper.

CASE 3.—When the dilutions taken are 1/2, 1/10, and 1/100, and the number of tubes taken are in the proportion 1 : 5 : 5.

In this case the values of the functions—

$$\frac{1}{\sqrt{\dfrac{E_{n/2}}{4} + \dfrac{E_{n/10}}{20} + \dfrac{E_{n/100}}{2000}}} = \frac{1}{\sqrt{K_3}} \quad \text{(say)} \dots\dots\dots\dots(6).$$

have been calculated for varying values of n and are presented in Table V. It may be remarked that this function is the measure of the standard error of n when for the dilution 1/2, 1/10, and 1/100 the number of tubes selected are 1, 5, and 5, respectively. The values of error are also shown in each case.

The values given in Table V cannot be compared with those in Tables I and III because, whereas the values in Table V relate to a total of eleven tubes in each test, the other two tables are based on a selection of three tubes only. In order to study the variations in the errors due to varying values of n and s Table VI is presented.

Variation in dilutions.—We have so far considered each of the three cases separately and have limited the discussion in each case to a chosen set of dilutions. A study of the previous tables reveals that for the same pair of values of n and s the errors may show different values. A few comparisons are presented below. The values of error of estimation for the two cases, 1 and 2, have been tabulated for a few values of n and s. Marked difference as well as similarity in the error of estimation may be noted.

Comparison of the error of estimation.

n.	s.	ERROR OF ESTIMATION.	
		Case 1.	Case 2.
2	1	222·3	142·80
20	20	24·60	24·83
500	500	6·00	10·86
1,000	1,000	4·13	47·14

The advantage gained in the reduction of error does not show a systematic variation from one set of dilutions to another. It is, therefore, of some interest to study how the error of estimation may be decreased by a deliberate selection of dilutions and by a proper choice of the number of tubes corresponding to each dilution taken for the test.

In order to clarify ideas it may be stated that each dilution chosen for the test serves two distinct purposes :—

    (i) Aids in the estimation of the probable number of organisms.

    (ii) Increases the accuracy of estimation by reducing the standard error of the probable number.

It is of interest to be able to assess numerically the effect any particular dilution exerts in the reduction of error. For this purpose, we may calculate, what has been called, ' the weight of determination of a dilution '. The greater the weight of a dilution the more advantageous will it be in increasing the reliability of the estimate of probable number. In terms of the previous notation the weight can be measured by the formula :—

$$\text{Weight of determination} = \frac{E_{n/a}x}{a^{2x}}$$

In designing experiments our object should be to choose only such dilutions as will provide us with the maximum weight of determination and should try and discard those dilutions which afford little or negligible help in increasing the reliability of our results. It will be clear from what follows that in particular cases, which can be determined from purely theoretical considerations, certain dilutions may be absolutely valueless.

Two dilutions $a = 2$ and $a = 10$ have been discussed in detail. Table VII presents for a few selected values of n the weight for a series of dilutions when the dilution factor $a = 2$.

If the dilution is expressed by $1/2^x$ it is usually assumed that x should take positive values such as 1, 2, 3, ..., i.e., we choose fractional dilutions. Theoretically it will be of interest to see what results will be obtained if in place of fractional dilutions we take multiples of 100 c.c. in each tube. For instance we may ask : what weight of determination will a dilution have if instead of 100 c.c. we took say 200 c.c. or 400 c.c. ? The idea has some theoretical basis to commend itself. Table VII shows that when in the original supply there is only 1 organism per 100 c.c. a more efficient method will be to take in each tube as much as 200 c.c. of the sample. It may be mentioned that tests on three different tubes containing 25 c.c., i.e., of $1/2^2$ dilution each, will provide the same reliability as one tube of 200 c.c. It does not, however, follow that if higher multiples of 100, say 400, 800 and so on, were taken greater reliability will be obtained. For higher multiples of 100 the weight rapidly decreases. Tube containing 100×2^4 c.c. provides no useful information. If 100 c.c. tubes were taken the weight will be 0·582, which shows only a small reduction from 0·626. On increasing the dilutions an adverse effect on the weight is observed. A tube of $1/2^{20}$ dilution again possesses a negligible value.

When in the same table we consider the higher probable numbers it is seen that the weight shows a definite fall. There is a general shift of maximum weight with increasing dilutions so that for n = 1,000 if at all, only $1/2^9$ dilution may be considered to provide the maximum weight.

Table VIII relates to the case when the dilution factor is 10 and sets out the weights of tubes for varying values of probable number and stage of dilution. In this case there appears to be no advantage gained by taking in any one tube

a larger volume than 100. For negative values of x the weights are small. This table also exhibits for increasing values of probable number a gradual shift of maximum weight towards higher dilutions. For instance, whereas for n = 1, x = 0 gives maximum weight, for n = 4 the maximum weight is obtained for x = 1, and so on.

It is of interest to investigate the relative weights obtained for different values of n in the three cases of dilution combinations previously discussed and to compare those with the maximum weight which it may theoretically be possible to attain by choosing only those dilutions which are most effective. In case 3 the number of tubes used is 11 as compared with three tubes used for cases 1 and 2. For this reason the weights in Table IX have been calculated per tube and are therefore comparable.

Table IX sets out for a few selected values of the probable number, the average weight of determination obtainable per tube for the three cases discussed previously. The maximum weight which would have been possible if proper dilution were selected is also shown. In the last three columns the weight actually obtained is expressed as a percentage of the maximum possible weight. A comparison of these three columns brings out important results. Case 2 has a marked superiority over cases 1 and 3 for small values of the probable number. For higher values of the probable number case 1 gives better results than case 2 or 3. It may be mentioned that case 2 includes dilutions 1/2, 1/10, and 1/100, whereas case 1 includes the dilutions 1/10, 1/100, and 1/100. For lower values of probable number of organisms the 1/2 dilution gives a marked superiority to case 2 while for higher values it is the 1/1,000 dilution which increases the efficiency of case 1. The main conclusion of practical importance which emerges from this study is that for those cases which usually arise in practice it will be advantageous to carry out a test by taking an equal number of tubes of 1/2, 1/10, and 1/100 dilutions. The extent of efficiency to be obtained by increasing the number of tubes may be seen from Table IV. Standard tables showing the estimated probable numbers of organisms for any combination of positive and negative results are under preparation.

SUMMARY.

Estimation of the probable number of organisms in a certain sample of water by the usual dilution test methods may give results which are unreliable. An investigation into the error involved in the determination has been carried out. The influence of the relevant factors has been studied in the case of the three most frequently adopted dilution tests. It has been shown that in practice it is advantageous to carry out a test by taking an equal number of tubes of 1/2, 1/10, and 1/100 dilutions.

REFERENCE.

SWAROOP, S. (1938) .. .. *Ind. Jour. Med. Res.*, **26**, 2, p. 353.

TABLE I.

Values of $\frac{1}{\sqrt{K_1}}$ *and* $\frac{100}{n\sqrt{K_1}}$ *for varying values of probable number n.*

Probable number n	$\frac{1}{\sqrt{K_1}}$	$\frac{100}{n\sqrt{K_1}}$	Probable number n	$\frac{1}{\sqrt{K_1}}$	$\frac{100}{n\sqrt{K_1}}$
(1)	(2)	(3)	(4)	(5)	(6)
1	3·071	307·10	28	31·748	113·31
2	4·445	222·25	29	33·086	114·09
3	5·573	185·77	30	34·449	114·83
4	6·589	164·73	40	49·124	122·81
5	7·545	150·90	50	64·350	128·70
6	8·468	141·13	60	78·409	130·68
7	9·372	133·89	70	90·698	129·57
8	10·268	128·35	80	101·554	126·94
9	11·164	124·04	90	111·557	123·95
10	12·065	120·65	100	121·19	121·13
11	12·975	117·95	120	140·02	116·69
12	13·898	115·82	140	159·322	113·80
13	14·836	114·12	160	179·433	112·15
14	15·793	112·81	180	200·506	111·39
15	16·769	111·79	200	222·671	111·34
16	17·767	111·04	225	252·017	112·01
17	18·787	110·51	250	283·260	113·30
18	19·833	110·18	275	316·407	115·06
19	20·903	110·02	300	351·410	117·14
20	21·999	110·00	350	426·399	121·83
21	23·122	110·10	400	506·442	126·61
22	24·272	110·33	500	671·172	134·23
23	25·450	110·65	600	826·221	137·70
24	26·655	111·06	700	963·280	137·61
25	27·887	111·55	800	108·929	135·62
26	29·147	112·10	900	1,197·456	133·05
27	30·435	112·72	1,000	1,305·795	130·58

TABLE II.

Values of error of estimation, viz., $\dfrac{\sigma_n}{n} \times 100$, *for varying number of tubes and different values of probable number.*

Probable number.	NUMBER OF TUBES.									
	1	3	5	10	20	50	100	200	500	1,000
1	307·10	177·30	137·34	97·11	68·67	43·43	30·71	21·71	13·73	9·71
2	222·25	128·32	99·39	70·28	49·70	31·43	22·23	15·70	9·95	7·00
3	185·77	107·27	83·08	58·75	41·54	26·27	18·57	13·13	8·30	5·87
5	150·90	87·12	67·48	47·79	33·74	21·34	15·10	10·66	6·74	4·76
10	120·65	69·66	53·96	38·15	26·98	17·06	12·07	8·53	5·39	3·81
15	111·79	64·54	49·99	35·35	25·00	15·81	11·18	7·91	5·00	3 53
20	110·00	63·51	49·19	34·78	24·60	15·56	·11·00	7·78	4·92	3·48
25	111·55	64·48	49·94	35·32	24·97	15·79	11·17	7·90	4·99	3·53
30	117·14	67·63	52·39	37·04	26·19	16·56	11·71	8·18	5·24	3·70
40	122·81	70·90	54·92	38·84	27·46	17·37	12·28	8·68	5·49	3·88
50	128·70	74·30	57·56	40·70	28·78	18·20	12·87	9·11	5·75	4·07
60	130·69	75·45	58·44	41·32	29·22	18·48	13·07	9·24	5·84	4·13
70	129·57	74·81	57·95	40·97	28·97	18·32	12·96	9·16	5·79	4·09
80	126·94	73·29	56·77	40·14	28·38	17·95	12·69	8·98	5·67	4·01
90	123·95	71·56	55·43	39·20	27·72	17·53	12 39	8·76	5·54	3·92
100	121·13	69·93	54·17	38·30	27·09	17·13	12·11	8·56	5·41	3·83
120	116·68	67·37	52·18	36·90	26·09	16·50	11·67	8·25	5·22	3·69
140	113·80	65·70	50·89	35·99	25·45	16·09	11·38	8·05	5·09	3·60
160	112·15	64·75	50·16	35·46	25·08	15·86	11·21	7·93	5·01	3·54
180	111·39	64·31	49·82	35·22	24·91	15·75	11·14	7·88	4·98	3·52
200	111·34	64·28	49·79	35·21	24·90	15·74	11·13	7·87	4·98	3·52
225	112·01	64·67	50·09	35·42	25·05	15·84	11·20	7·92	5·01	3·54
250	113·31	65·41	50·67	35·83	25·33	16·02	11·33	8·01	5·06	3·58
275	115·06	66·43	54·46	36·39	25·73	16·27	11·51	8·13	5·14	3·64

TABLE II—*concld.*

Probable number.	NUMBER OF TUBES.									
	1	3	5	10	20	50	100	200	500	1,000
300	114·83	67·63	52·39	37·04	26·19	16·56	11·71	8·28	5·24	3·70
350	121·83	70·34	54·48	38·53	27·24	17·23	12·18	8·61	5·45	3·85
400	126·61	73·10	56·62	40·04	28·31	17·90	12·66	8·95	5·66	4·00
500	134·23	77·50	60·03	42·45	30·01	18·98	13·42	9·49	6·00	4·24
600	137·70	79·51	61·58	43·54	30·79	19·47	13·77	9·74	6·16	4·35
700	137·61	79·45	61·54	43·52	30·77	19·46	13·76	9·73	6·15	4·35
800	135·62	78·30	60·65	42·89	30·33	19·18	13·56	9·59	6·06	4·29
900	133·05	76·82	59·50	42·07	29·75	18·81	13·30	9·42	5·95	4·20
1,000	130·58	75·39	58·40	41·29	29·20	18·46	13·06	9·23	5·84	4·13

TABLE III.

Values of $\dfrac{1}{\sqrt{K_2}}$ *and* $\dfrac{100}{n\sqrt{K_2}}$ *for varying values of probable number.*

Probable number n	$\dfrac{1}{\sqrt{K_2}}$	$\dfrac{100}{n\sqrt{K_2}}$	Probable number n	$\dfrac{1}{\sqrt{K_2}}$	$\dfrac{100}{n\sqrt{K_2}}$
(1)	(2)	(3)	(4)	(5)	(6)
1	1·43	142·80	11	12·06	109·64
2	2·26	113·05	12	13·23	110·23
3	3·11	103·53	13	14·37	110·53
4	4·02	100·48	14	15·49	110·66
5	5·02	100·35	15	16·60	110·67
6	6·10	101·66	16	17·71	110·66
7	7·25	103·56	17	18·81	110·66
8	8·44	105·55	18	19·93	110·71
9	9·66	107·31	19	21·06	110·83
10	10·87	108·68	20	22·21	111·03

TABLE III—*concld.*

Probable number n	$\dfrac{1}{\sqrt{K_2}}$	$\dfrac{100}{n\sqrt{K_2}}$	Probable number n	$\dfrac{1}{\sqrt{K_2}}$	$\dfrac{100}{n\sqrt{K_2}}$
(1)	(2)	(3)	(4)	(5)	(6)
21	23·37	111·30	120	152·23	126·86
22	24·57	111·67	140	174·79	124·85
23	25·79	112·13	160	198·85	124·28
24	27·04	112·67	180	224·72	124·84
25	28·32	113·26	200	252·77	126·39
26	29·62	113·93	225	291·34	129·48
27	30·96	114·66	250	334·40	133·76
28	32·32	115·44	275	382·66	139·15
29	33·72	116·28	300	436·87	145·62
30	35·14	117·14	350	566·73	161·92
40	50·64	126·61	400	732·12	183·03
50	67·12	134·23	500	1,214·18	242·84
60	82·62	137·71	600	2,006·06	334·34
70	96·33	137·61	700	3,309·61	472·80
80	108·50	135·63	800	5,455·53	681·94
90	119·75	133·06	900	9,017·13	1,001·90 ·
100	130·58	130·58	1,000	14,907·57	1,490·76

TABLE IV.

Showing the error in estimation for various values of the probable number and the numbers of tubes employed.

Probable number.	NUMBER OF TUBES.									
	1	3	5	10	20	50	100	200	500	1,000
1	142·80	82·45	63·86	45·16	31·93	20·19	14·28	10·10	6·39	4·52
2	113·05	65·27	50·56	35·75	25·28	15·99	11·31	7·99	5·06	3·57
3	103·53	59·77	46·30	32·74	23·15	14·64	10·35	7·32	4·63	3·27
5	100·35	57·94	44·88	31·73	22·44	14·19	10·04	7·10	4·49	3·17
10	108·68	62·75	48·60	34·37	24·30	15·37	10·87	7·68	4·86	3·44
15	110·67	63·90	49·49	35·00	24·75	15·65	11·07	7·83	4·95	3·50

TABLE IV—*concld.*

Probable number.	NUMBER OF TUBES.									
	1	3	5	10	20	50	100	200	500	1,000
20	111·03	64·10	49·65	35·11	24·83	15·70	11·10	7·85	4·97	3·51
25	113·26	65·39	50·65	35·82	25·33	16·02	11·33	8·01	5·07	3·58
30	117·14	67·63	52·39	37·04	26·19	16·57	11·71	8·28	5·24	3·70
40	126·61	73·10	56·62	40·04	28·31	17·91	12·66	8·95	5·66	4·00
50	134·23	77·50	60·03	42·45	30·01	18·98	13·42	9·49	6·00	4·24
60	137·71	79·51	61·59	43·55	30·79	19·48	13·77	9·74	6·16	4·35
70	137·61	79·45	61·54	43·52	30·77	19·46	13·76	9·73	6·15	4·35
80	135·61	78·29	60·65	42·88	30·32	19·18	13·56	9·59	6·06	4·29
90	133·06	76·82	59·51	42·08	29·75	18·82	13·31	9·41	5·95	4·21
100	130·58	75·39	58·40	41·29	29·20	18·47	13·06	9·23	5·81	4·13
120	126·84	73·23	56·72	40·11	28·36	17·91	12·68	8·97	5·67	4·01
140	124·83	72·07	55·83	39·47	27·91	17·65	12·48	8·83	5·58	3·95
160	124·41	71·83	55·64	39·34	27·82	17·59	12·44	8·80	5·56	3·93
180	124·84	72·08	55·83	39·48	27·92	17·66	12·48	8·83	5·58	3·95
200	126·38	72·97	56·52	39·96	28·26	17·87	12·64	8·94	5·65	4·00
225	129·48	74·76	57·91	40·95	28·95	18·31	12·95	9·16	5·79	4·09
250	133·76	77·23	59·82	42·30	29·91	18·92	13·38	9·46	5·98	4·23
275	139·15	80·34	62·23	44·00	31·11	19·68	13·92	9·84	6·22	4·40
300	145·62	84·07	65·12	46·05	32·56	20·59	14·56	10·30	6·51	4·60
350	161·91	93·48	72·41	51·20	36·20	22·90	16·19	11·45	7·24	5·12
400	183·03	105·67	81·85	57·88	40·93	25·88	18·30	12·94	8·19	5·79
500	242·82	110·19	108·59	76·79	54·30	34·34	24·28	17·17	10·86	7·68
600	334·33	193·03	149·52	105·72	74·76	47·28	33·43	23·64	14·95	10·57
700	472·79	272·97	211·44	149·51	105·72	66·86	47·28	33·43	21·14	14·95
800	681·94	393·72	304·97	215·65	152·49	96·44	68·19	48·22	30·50	21·56
900	1,001·86	578·42	448·05	316·82	224·02	141·68	100·19	70·84	44·80	31·68
1,000	1,490·70	860·66	666·66	471·40	333·33	210·82	149·07	105·41	66·67	47·14

TABLE V.

Values of $\sqrt{\dfrac{1}{K_3}}$ *and* $\dfrac{100}{n\sqrt{K_3}}$ *for varying values of probable number.*

n	$\dfrac{1}{\sqrt{K_3}}$	$\dfrac{100}{n\sqrt{K_3}}$	n	$\dfrac{1}{\sqrt{K_3}}$	$\dfrac{100}{n\sqrt{K_3}}$
(1)	(2)	(3)	(4)	(5)	(6)
1	1·05	104·80	24	12·10	50·40
2	1·59	79·45	25	12·67	50·67
3	2·08	69·33	26	13·25	50·96
4	2·56	63·91	27	13·85	51·29
5	3·03	60·53	28	14·46	51·63
6	3·49	58·20	29	15·08	52·00
7	3·95	56·47	30	15·72	52·38
8	4·41	55·10	40	22·65	56·62
9	4·86	53·98	50	30·05	60·10
10	5·30	53·04	60	36·95	61·58
11	5·75	52·25	70	43·08	61·54
12	6·19	51·59	80	48·52	60·65
13	6·64	51·04	90	53·55	59·50
14	7·09	50·61	100	58·40	58·40
15	7·54	50·27	120	68·07	56·73
16	8·00	50·02	140	78·16	55·83
17	8·47	49·85	160	88·91	55·57
18	8·96	49·75	180	100·50	55·83
19	9·45	49·73	200	113·04	56·52
20	9·96	49·82	225	130·29	57·91
21	10·47	49·85	250	149·55	59·82
22	11·00	50·00	275	171·13	62·23
23	11·54	50·18	300	195·37	65·12

TABLE V—*concld.*

n	$\dfrac{1}{\sqrt{K_3}}$	$\dfrac{100}{n\sqrt{K_3}}$	n	$\dfrac{1}{\sqrt{K_3}}$	$\dfrac{100}{n\sqrt{K_3}}$
(1)	(2)	(3)	(4)	(5)	(6)
350	253·44	72·41	700	1,480·06	211·44
400	327·41	81·85	800	2,439·75	304·97
500	542·97	108·59	900	4,032·39	448·04
600	897·12	149·52	1,000	6,666·67	666·67

TABLE VI.

Showing the error in estimation for varying values of probable number and for a few multiples of such combinations of tubes as are in the proportion 1 : 5 : 5 for dilutions 1/2, 1/10, and 1/100, respectively.

Probable number n	RESPECTIVE NUMBERS OF TUBES CHOSEN FOR THE DILUTIONS 1/2, 1/10, AND 1/100.									
	1: 5: 5.	3: 15: 15.	5: 25: 25.	10: 50: 50.	20: 100: 100.	50: 250: 250.	100: 500: 500.	200: 1,000: 1,000.	500: 2,500: 2,500.	1,000: 5,000: 5,000.
1	104·80	60·50	46·87	33·14	23·43	14·82	10·48	7·41	4·69	3·31
2	79·45	45·87	35·53	25·12	17·77	11·23	7·95	5·62	3·55	2·51
3	69·33	40·03	31·00	21·92	15·50	9·80	6·93	4·90	3·10	2·19
5	60·53	34·95	27·07	19·14	13·53	8·56	6·05	4·28	2·71	1·91
10	53·04	30·63	23·72	16·77	11·86	7·50	5·30	3·75	2·37	1·68
15	50·27	29·03	22·48	15·90	11·24	7·11	5·03	3·55	2·25	1·59
20	49·82	28·77	22·28	15·75	11·14	7·04	4·98	3·52	2·23	1·57
25	50·67	29·26	22·66	16·02	11·33	7·16	5·07	3·58	2·26	1·60

TABLE VI—*concld.*

Probable number n	RESPECTIVE NUMBERS OF TUBES CHOSEN FOR THE DILUTIONS 1/2, 1/10, AND 1/100.									
	1 : 5 : 5.	3 : 15 : 15.	5 : 25 : 25.	10 : 50 : 50.	20 : 100 : 100.	50 : 250 : 250.	100 : 500 : 500.	200 : 1,000 : 1,000.	500 : 2,500 : 2,500.	1,000 : 5,000 : 5,000.
30	52·38	30·24	23·42	16·56	11·71	7·40	5·24	3·70	2·34	1·66
40	56·62	32·69	25·32	17·90	12·66	8·01	5·66	4·00	2·53	1·79
50	60·10	34·70	26·88	19·00	13·44	8·50	6·01	4·25	2·69	1·90
60	61·58	35·56	27·54	19·47	13·77	8·71	6·16	4·35	2·75	1·95
70	61·54	35·53	27·52	19·46	13·76	8·70	6·15	4·35	2·75	1·94
80	60·65	35·02	27·12	19·18	13·56	8·58	6·07	4·29	2·71	1·92
90	59·50	34·35	26·61	18·81	13·30	8·41	5·95	4·21	2·66	1·88
100	58·40	33·72	26·12	18·47	13·06	8·26	5·84	4·13	2·61	1·85
120	56·73	32·76	25·37	17·94	12·68	8·02	5·67	4·01	2·54	1·79
140	55·83	32·24	24·97	17·65	12·48	7·89	5·58	3·95	2·50	1·76
160	55·57	32·09	24·85	17·57	12·43	7·86	5·56	3·93	2·48	1·76
180	55·83	32·24	24·97	17·65	12·48	7·89	5·58	3·95	2·50	1·76
200	56·52	32·63	25·28	17·87	12·64	7·99	5·65	3·99	2·53	1·79
225	57·91	33·44	25·90	18·31	12·95	8·19	5·79	4·09	2·59	1·83
250	59·54	34·54	26·75	18·92	13·37	8·46	5·98	4·23	2·67	1·89
275	62·23	35·93	27·83	19·68	13·91	8·80	6·22	4·40	2·78	1·97
300	65·12	37·60	29·12	20·59	14·56	9·21	6·51	4·60	2·91	2·06
350	72·41	41·81	32·38	22·90	16·19	10·24	7·24	5·12	3·24	2·29
400	81·85	47·26	36·60	25·88	18·30	11·57	8·19	5·79	3·66	2·59
500	108·59	62·70	48·56	34·34	24·28	15·35	10·86	7·68	4·85	3·43
600	149·52	86·33	66·87	47·28	33·43	21·14	14·95	10·57	6·68	4·72
700	211·14	122·09	94·56	66·86	47·28	29·90	21·14	14·95	9·45	6·68
800	304·97	176·09	136·38	96·43	68·19	43·12	30·50	21·56	13·63	9·64
900	448·04	258·70	200·36	141·67	100·18	63·35	44·80	31·68	20·03	14·16
1,000	666·67	384·94	298·13	210·80	149·07	94·27	66·67	47·13	29·80	21·67

TABLE VII.

Weight of determination $\left(=\dfrac{E_{n|2^x}}{4^x}\right)$ when the dilution factor $a = 2$.

PROBABLE NUMBER N.

Dilution x	1	3	5	10	15	20	30	50	100	500	1,000
−4	0·021504	0·000000									
−3	0·298512	0·000096	0·000000								
−2	0·626072	0·009940	0·000180	0·000000							
−1	0·581977	0·053396	0·006784	0·000045	0·000000						
0	0·385374	0·071804	0·022356	0·001696	0·000138	0·000011					
1	0·220051	0·055953	0·025097	0·005589	0·001505	0·000424	0·000000				
2	0·117350	0·034341	0·017996	0·006274	0·002830	0·001397	0·000035				
3	0·060562	0·018935	0·010650	0·004499	0·002514	0·001569	0·000376	0·000030	0·000000		
4	0·030806	0·009934	0·005775	0·002662	0·001633	0·001125	0·000707	0·000180	0·000008		
5	0·015550	0·005086	0·003003	0·001444	0·000924	0·000665	0·000629	0·000259	0·000045		
6	0·007826	0·002576	0·001534	0·000751	0·000491	0·000361	0·000408	0·000206	0·000065	0·000000	
7	0·003913	0·001293	0·000775	0·000383	0·000253	0·000188	0·000231	0·000128	0·000052	0·000001	
8	0·001905	0·000646	0·000389	0·000193	0·000128	0·000096	0·000123	0·000071	0·000032	0·000003	
9	0·000954	0·000329	0·000194	0·000097	0·000064	0·000048	0·000063	0·000037	0·000018	0·000002	0·000001
10	0·000476	0·000159	0·000099	0·000049	0·000032	0·000024	0·000032	0·000019	0·000009	0·000002	0·000001
11	0·000246	0·000086	0·000050	0·000025	0·000016	0·000012	0·000016	0·000010	0·000005	0·000001	
12	0·000125	0·000038	0·000025	0·000013	0·000008	0·000006	0·000008	0·000005	0·000002		
13	0·000067	0·000022	0·000013	0·000007	0·000004	0·000003	0·000004	0·000003	0·000001		
14	0·000033	0·000010	0·000007	0·000003	0·000002	0·000002	0·000002	0·000001			
15	0·000012	0·000006	0·000003	0·000002	0·000001	0·000001	0·000001				
16	0·000007	0·000003	0·000002	0·000001							
17	0·000003	0·000002	0·000001								
18	0·000002	0·000001									
19	0·000001										
20											

TABLE VIII.

Weight of determination $\left(= \dfrac{E_{n/10^x}}{10^{2x}} \right)$ *when the dilution factor* a $= 10$.

Dilution x	PROBABLE NUMBER N.							
	1	2	3	4	5	6	7	8
−2	0·000000	..	..	..	..	..	..	..
−1	0·004500	..	..	..	..	..	..	..
0	0·581977	0·156518	0·052396	0·018657	0·006784	0·002485	0·000913	0·000336
1	0·095083	0·045167	0·028583	0·020332	0·015415	0·012164	0·009864	0·008160
2	0·009950	0·004950	0·003284	0·002450	0·001950	0·001617	0·001379	0·001201
3	0·001000	0·000500	0·000333	0·000250	0·000200	0·000166	0·000142	0·000125
4	0·000093	0·000050	0·000033	0·000025	0·000020	0·000017	0·000014	0·000012
5	0·000010	0·000005	0·000003	0·000003	0·000002	0·000002	0·000001	0·000001
6	0·000000	..	..	..	..	..	..	..

	9	10	15	20	30	50	100	500
−2	..	..	..	..	..	..	..	..
−1	..	..	..	..	..	..	..	..
0	0·000123	0·000045	0·000000	..	..	..	..	..
1	0·006851	0·005820	0·002872	0·001565	0·000524	0·000068	..	..
2	0·001062	0·000951	0·000618	0·000452	0·000286	0·000154	0·000058	0·000001
3	0·000111	0·000100	0·000066	0·000050	0·000033	0·000020	0·000010	0·000002
4	0·000011	0·000010	0·000007	0·000005	0·000003	0·000002	0·000001	..
5	0·000001	0·000001	..	..	..	..	..	..
6	..	..	..	..	..	..	..	..

TABLE IX.

*Comparison of the weight of determination for three different cases of
dilutions discussed in the text.*

Probable number.	WEIGHT OF DETERMINATION PER TUBE.						Weight actually obtained expressed as a percentage of the maximum possible weight.		
	CASE 1.		CASE 2.		CASE 3.		Case 1.	Case 2.	Case 3.
	Weight actually obtained.	Maximum possible weight.	Weight actually obtained.	Maximum possible weight.	Weight actually obtained.	Maximum possible weight.			
1	0·035344	0·581977	0·163469	0·626072	0·044582	0·626072	6·07	26·11	7·12
3	0·010733	0·052396	0·034557	0·071804	0·009424	0·071804	20·48	48·13	13·12
5	0·005855	0·015415	0·013240	0·025097	0·003611	0·025097	37·98	52·76	14·39
10	0·002290	0·005820	0·002822	0·006274	0·000770	0·006274	39·35	44·98	12·27
15	0·001185	0·002872	0·001209	0·002872	0·000330	0·002872	41·26	42·10	11·49
20	0·000689	0·001565	0·000676	0·001569	0·000184	0·001569	44·03	43·08	11·73
30	0·000281	0·000524	0·000270	0·000707	0·000074	0·000707	53·63	38·19	10·47
50	0·000081	0·000154	0·000074	0·000154	0·000020	0·000154	52·60	48·05	12·99
100	0·000023	0·000058	0·000019	0·000045	0·000005	0·000045	39·66	42·22	11·11

SIZE OF SAMPLES NECESSARY FOR ESTABLISHING SIGNIFICANCE OF THE DIFFERENCE IN RESPONSES TO TWO DIFFERENT TREATMENTS.

BY

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IN biological experiments the results obtained by two methods of treatment, such as the administration of two drugs A and B, to two groups of a given size of randomly selected animals are not always easy of interpretation. The question which has to be answered is whether the difference in the responses such as those observed could have arisen from pure chance or whether it could reasonably be attributed to the difference in the values of the drugs. The degree of the reliability of the difference will naturally vary and its significance can only be arbitrarily fixed. The statistician answers the question by stating that such a difference or. a difference greater than this could have arisen from pure chance only in 1 in 100 or in 5 in 100, etc., cases. More briefly he states that the probability is 0·01 or 0·05, etc. Obviously the smaller the probability the greater is the confidence which can be placed in the difference being due to difference in the actions of the drugs. It is for the experimentalist to decide at what level of significance he will consider his results reliable. While non-significance of the results will make it unjustifiable to conclude that the two drugs are different in their action it is possible that this result may be obtained even when the drugs are really different because the number of animals experimented upon was insufficient. It is, therefore, necessary to calculate the size of the samples which is required in order to have the assurance that the given responses are significantly different.

It will be observed that the sufficiency of the number is determined on the basis of the knowledge of the two percentages. If these two percentages are very nearly equal we require a very large number of animals in each sample to establish the significance of the difference. The converse also holds good. As an example, if we want to bring out significance of the difference between 28 per cent and 32 per cent both the samples have to contain more than 1,000 animals. On the other hand samples of size 70 will suffice to establish a significant difference between 28 per cent and 50 per cent. Unless, therefore, there is some indication of the magnitude of the difference from preliminary trials or on *a priori* grounds

it is not possible to calculate the size of the samples required to produce significant results. But in one particular case the problem shows itself clearly. Drug A has been in use for reducing mortality from a particular disease for a long time. The hospital records give a figure for case mortality of say 30 per cent for this drug, which is based on a very large number of observations. Another drug B is discovered and it is claimed that this drug B reduces case mortality to 10 per cent. In such a case as this we know the two percentages in advance and can therefore calculate the number of animals on which the two drugs should be tried to test the claim satisfactorily.

Methods for approximately calculating such numbers have been in use and the one most commonly used has been the so-called npq method. The value of n, i.e., the size of the sample which will give a significant difference between x per cent and y per cent on the 1 per cent probability level, can be calculated from the following formula—

$$\frac{x - y}{\sqrt{\frac{(x + y)\ (200 - x - y)}{2n}}} = 2 \cdot 576$$

For example, if 20 per cent and 10 per cent are to be compared $x = 20$, $y = 10$ and the value of n is given by—

$$\frac{20 - 10}{\sqrt{\frac{(20 + 10)\ (200 - 30)}{2n}}} = 2 \cdot 576, \text{ or } n = 169.$$

According to this method therefore the minimum number of animals in each random sample will be 169. It is by this method that Ross and Stott (1911) calculated their significance test tables. This method, however, is approximate and in many cases yields very erroneous results. This is because the test is based on certain theoretical assumptions which, as a rule, are not satisfied when the percentages compared are small or the size of samples on which percentages are based is small.

The tables now presented have been calculated on the basis of an exact formula which Fisher (1936) has given for the calculation of the exact value of probability from four-fold tables. If n is the size of the samples and a and b are the numbers producing the desired responses after the administration of drugs A and B respectively, then the information may be tabulated as follows :—

	Response noted.	Response not noted.	Total.
Drug A ..	a	n—a	n
Drug B ..	b	n—b	n
	a + b	2n—a—b	2n

The probability P that this combination of a and b arose purely due to chance and not due to any difference between the two drugs is—

$$P = \frac{(n!)^2 \, (a + b)! \, (2n - a - b)!}{2n! \, a! \, b! \, (n - a)! \, (n - b)!}.$$

In the same way we can calculate the probability of the occurrence of more divergent cases due to chance alone and by summing up all these values of P we can find the probability that a combination like a, b, or more unlikely combinations arose from errors of random sampling[*]. If this accumulated value of P is 0·01 or less the result is taken as significant, i.e., not likely to be due to chance. Some workers may prefer a less stringent level of 0·05.

For our purpose we have to solve the converse. The value of the summed up probability, in a case such as given above, being 0·01 what different values should n assume against various combinations of responses to the two drugs? These numbers have been calculated for all combinations of percentages which are significantly different when the size does not exceed 100. A perusal of the tables will show that only alternate percentages have been compared. Owing to laborious calculations involved it has not been possible to extend these tables to higher sizes and remaining percentages[†].

The use of the tables may be illustrated by an example: It is known from previous experience that after the administration of drug A the case mortality from a particular disease is 60 per cent. It is claimed that a new drug B can reduce the mortality to 30 per cent. How many experimental animals are necessary for the test? The table shows that, against the pair of values 30 per cent and 60 per cent samples of 30 correspond to P = 0·01850 and those of size 40 to P = 0·00646. Therefore, at least 30 to 40 animals should be used in the test.

It should, however, be noted that the difference in percentages when significant can be ascribed to the action of the drugs only when all other sources which might contribute towards it have been eliminated. This criterion is, as a rule, difficult to satisfy and therefore, in arriving at a conclusion, due caution should be exercised.

ACKNOWLEDGMENTS.

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Ross, R., and STOTT, W. (1911) .. *Annals Trop. Med. & Parasit.*, **5**, No. 3, p. 347.

[*] It is usual to consider that the value a may be associated with drug B and b with drug A. This possibility would be taken into consideration only when we do not know in advance that the percentage of recovery from the experimental drug is higher than that from the control.

[†] More comprehensive tables for 0·01 and 0·05 levels of significance are under preparation.

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment.

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.		Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
0	6	110	0·00708	100	0·01447	2	30	30	0·00609	20	0·01010
0	8	90	0·00692	80	0·01418	2	32	30	0·00279	20	0·01010
0	10	70	0·00668	60	0·01371	2	34	20	0·00416	10	0·10526
0	12	60	0·00649	50	0·01333						
0	14	50	0·00624	40	0·01277	4	16	100	0·00404	90	0·01153
0	16	50	0·00289	40	0·01277	4	18	60	0·00794	50	0·02556
0	18	40	0·00587	30	0·02609	4	20	60	0·00419	50	0·01387
0	20	40	0·00265	30	0·01186	4	22	50	0·00734	40	0·02381
0	22	30	0·00527	20	0·05301	4	24	50	0·00379	40	0·01260
0	24	30	0·00527	20	0·02356	4	26	50	0·00191	40	0·01260
0	26	30	0·00229	20	0·02356	4	28	40	0·00647	30	0·01285
0	28	30	0·00229	20	0·01010	4	30	30	0·00609	20	0·04574
0	30	30	0·00097	20	0·01010	4	32	30	0·00279	20	0·04574
0	32	30	0·00040	20	0·01010	4	34	30	0·00279	20	0·02180
0	34	20	0·00416	10	0·10526	4	36	30	0·00123	20	0·02180
						4	38	20	0·00983	10	0·04334
2	10	110	0·00938	100	0·01652						
2	12	90	0·00899	80	0·01585	6	0	110	0·00708	100	0·01447
2	14	70	0·00442	60	0·01609	6	18	90	0·00924	·80	0·02415
2	16	60	0·00419	50	0·01543	6	20	80	0·00877	70	0·01049
2	18	50	0·00783	40	0·02838	6	22	70	0·00598	60	0·01694
2	20	50	0·00389	40	0·01444	6	24	60	0·00973	50	0·01130
2	22	40	0·00716	30	0·02616	6	26	50	0·00611	40	0·01260
2	24	40	0·00346	30	0·02616	6	28	40	0·00647	30	0·03986
2	26	40	0·00346	30	0·01285	6	30	40	0·00322	30	0·02095
2	28	40	0·00163	30	0·01285	6	32	40	0·00156	30	0·01057

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
6	34	40	0·00073	30	0·01057	10	30	60	0·00550	50	0·01142
6	36	30	0·00512	20	0·02180	10	32	50	0·00641	40	0·01343
6	38	20	0·00983	10	0·15170	10	34	40	0·00724	30	0·02873
8	0	90	0·00692	80	0·01418	10	36	40	0·00724	30	0·01517
8	20	110	0·00947	100	0·01183	10	38	40	0·00377	30	0·01517
8	22	80	0·00680	70	0·02820	10	40	30	0·00767	20	0·03242
8	24	80	0·00400	70	0·01053	10	42	30	0·00370	20	0·03242
S	26	60	0·00742	50	0·01551	10	44	30	0·00370	20	0·01548
S	28	50	0·00870	40	0·01837	10	46	30	0·00171	20	0·01548
8	30	40	0·00988	30	0·02095	10	48	20	0·00691	10	0·07044
8	32	40	0·00514	30	0·01057	12	0	60	0·00649	50	0·01333
8	34	40	0·00259	30	0·01057	12	2	90	0·00899	80	0·01585
8	36	30	0·00512	20	0·06369	12	26	100	0·00916	90	0·01758
8	38	30	0·00512	20	0·03242	12	28	70	0·00952	60	0·01926
S	40	30	0·00238	20	0·03242	12	30	70	0·00573	60	0·01166
S	42	30	0·00106	20	0·03242	12	32	60	0·00689	50	0·01417
8	44	30	0·00106	20	0·01548	12	34	50	0·00815	40	0·01695
8	46	30	0·00045	20	0·01548	12	36	50	0·00456	40	0·01695
8	48	20	0·00691	10	0·07044	12	38	40	0·00939	30	0·03581
10	0	70	0·00668	60	0·01371	12	40	40	0·00502	30	0·01955
10	2	110	0·00938	100	0·01652	12	42	40	0·00259	30	0·01019
10	24	90	0·00842	80	0·01671	12	44	40	0·00129	30	0·01019
10	26	80	0·00642	70	0·01297	12	46	30	0·00506	20	0·01548
10	28	60	0·00951	50	0·01976	12	48	20	0·00691	10	0·07044

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
14	0	50	0·00624	40	0·01277	16	42	40	0·00627	30	0·02351
14	2	70	0·00442	60	0·01609	16	44	40	0·00330	30	0·02351
14	28	110	0·00617	100	0·01164	16	46	40	0·00330	30	0·01253
14	30	90	0·00953	80	0·01045	16	48	40	0·00167	30	0·01253
14	32	80	0·00406	70	0·01294	16	50	30	0·00635	20	0·02037
14	34	60	0·00831	50	0·01689	16	52	30	0·00305	20	0·02037
14	36	50	0·00991	40	0·03458	16	54	20	0·00935	10	0·17492
14	38	50	0·00565	40	0·02030	18	0	40	0·00587	30	0·02609
14	40	50	0·00313	40	0·01150	18	2	50	0·00783	40	0·02838
14	42	40	0·00627	30	0·01019	18	4	60	0·00794	50	0·02556
14	44	40	0·00330	30	0·01019	18	6	90	0·00924	80	0·02415
14	46	30	0·00506	20	0·04118	18	34	90	0·00849	80	0·01453
14	48	30	0·00506	20	0·02037	18	36	80	0·00601	70	0·01789
14	50	30	0·00239	20	0·02037	18	38	70	0·00722	60	0·01253
14	52	30	0·00108	20	0·02037	18	40	60	0·00765	50	0·01330
14	54	20	0·00935	10	0·07044	18	42	50	0·00781	40	0·01350
16	0	50	0·00289	40	0·01277	18	44	40	0·00749	30	0·02351
16	2	60	0·00419	50	0·01543	18	46	40	0·00749	30	0·01253
16	4	100	0·00404	90	0·01153	18	48	40	0·00400	30	0·01253
16	32	90	0·00691	80	0·01317	18	50	30	0·00635	20	0·04792
16	34	70	0·00921	60	0·02836	18	52	30	0·00305	20	0·04792
16	36	70	0·00567	60	0·01116	18	54	30	0·00305	20	0·02419
16	38	60	0·00674	50	0·01164	18	56	30	0·00139	20	0·02419
16	40	50	0·00674	40	0·01150	18	58	30	0·00139	20	0·01124

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		HIGHER VALUE or N.		LOWER VALUE OF N.		Comparison between, per cent.		HIGHER VALUE or N.		LOWER VALUE or N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
18	60	30	0·00060	20	0·01124	22	0	30	0·00527	20	0·05301
18	62	30	0·00024	20	0·01124	22	2	40	0·00716	30	0·02616
18	64	20	0·00477	10	0·08491	22	4	50	0·00734	40	0·02381
						22	6	70	0·00598	60	0·01694
20	0	40	0·00265	30	0·01186	22	8	80	0·00680	70	0·02820
20	2	50	0·00389	40	0·01444	22	38	110	0·00604	100	0·01012
20	4	60	0·00419	50	0·01387	22	40	90	0·00767	80	0·01305
20	6	80	0·00877	70	0·01049	22	42	70	0·00871	60	0·01509
20	8	110	0·00947	100	0·01183	22	44	60	0·00938	50	0·01633
20	36	100	0·00886	90	0·01498	22	46	50	0·00978	40	0·02878
20	38	90	0·00661	80	0·01130	22	48	50	0·00567	40	0·01699
20	40	70	0·00798	60	0·01385	22	50	40	0·00964	30	0·02980
20	42	60	0·00853	50	0·01487	22	52	40	0·00525	30	0·01630
20	44	50	0·00883	40	0·01534	22	54	40	0·00274	30	0·01630
20	46	50	0·00508	40	0·01534	22	56	30	0·00843	20	0·02419
20	48	40	0·00862	30	0·02695	22	58	30	0·00843	20	0·01124
20	50	40	0·00466	30	0·01459	22	60	30	0·00411	20	0·01124
20	52	30	0·00749	20	0·04792	22	62	30	0·00188	20	0·01124
20	54	30	0·00749	20	0·02419	22	64	20	0·00477	10	0·08491
20	56	30	0·00364	20	0·02419						
20	58	30	0·00364	20	0·01124	24	0	30	0·00527	20	0·02356
20	60	30	0·00166	20	0·01124	24	2	40	0·00346	30	0·02616
20	62	30	0·00072	20	0·01124	24	4	50	0·00379	40	0·01260
20	64	20	0·00477	10	0·08491	24	6	60	0·00973	50	0·01130

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
24	8	80	0·00400	70	0·01053	26	48	70	0·00420	60	0·01157
24	10	90	0·00842	80	0·01671	26	50	60	0·00712	50	0·01142
24	42	80	0·00914	70	0·02360	26	52	50	0·00670	40	0·01051
24	44	80	0·00590	70	0·01008	26	54	40	0·00575	30	0·03204
24	46	60	0·00620	50	0·01765	26	56	40	·0·00575	30	0·01761
24	48	60	0·00367	50	0·01065	26	58	40	0·00301	30	0·01761
24	50	50	0·00622	40	0·01842	26	60	30	0·00912	20	0·02678
24	52	50	0·00351	40	0·01051	26	62	30	0·00443	20	0·02678
24	54	40	0·00575	30	0·01630	26	64	30	0·00443	20	0·01242
24	56	30	0·00843	20	0·05267	26	66	30	0·00201	20	0·01242
24	58	30	0·00843	20	0·02678	26	68	20	0·00519	10	0·08945
24	60	30	0·00411	20	0·02678	28	0	30	0·00229	20	0·01010
24	62	30	0·00188	20	0·02678	28	2	40	0·00163	30	0·01285
24	64	30	0·00188	20	0·01242	28	4	40	0·00647	30	0·01285
24	66	30	0·00080	20	0·01242	28	6	40	0·00647	30	0·03986
24	68	20	0·00519	10	0·03489	28	8	50	0·00870	40	0·01837
26	0	30	0·00229	20	0·02356	28	10	60	0·00951	50	0·01976
26	2	40	0·00346	30	0·01285	28	12	70	0·00952	60	0·01926
26	4	50	0·00191	40	0·01260	28	14	110	0·00617	100	0·01164
26	6	50	0·00611	40	0·01260	28	46	100	0·00627	90	0·01000
26	8	60	0·00742	50	0·01551	28	48	80	0·00699	70	0·01179
26	10	80	0·00642	70	0·01297	28	50	70	0·00753	60	0·01217
26	12	100	0·00916	90	0·01758	28	52	60	0·00751	50	0·01209
26	44	90	0·00606	80	0·01537	28	54	50	0·00711	40	0·01124
26	46	80	0·00665	70	0·01070	28	56	50	0·00404	40	0·01124

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.		Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
28	58	40	0·00616	30	0·01761	30	64	30	0·00955	20	0·02808
28	60	30	0·00912	20	0·05548	30	66	30	0·00460	20	0·02808
28	62	30	0·00443	20	0·05548	30	68	30	0·00460	20	0·01282
28	64	30	0·00443	20	0·02808	30	70	30	0·00206	20	0·01282
28	66	30	0·00201	20	0·02808	30	72	30	0·00085	20	0·01282
28	68	30	0·00201	20	0·01282	30	74	20	0·00519	10	0·08945
28	70	30	0·00085	20	0·01282	32	0	30	0·00040	20	0·01010
28	72	30	0·00033	20	0·01282	32	2	30	0·00279	20	0·01010
28	74	20	0·00519	10	0·08945	32	4	30	0·00279	20	0·04574
30	0	30	0·00097	20	0·01010	32	6	40	0·00156	30	0·01057
30	2	30	0·00609	20	0·01010	32	8	40	0·00514	30	0·01057
30	4	30	0·00609	20	0·04574	32	10	50	0·00641	40	0·01343
30	6	40	0·00322	30	0·02095	32	12	60	0·00689	50	0·01417
30	8	40	0·00988	30	0·02095	32	14	80	0·00406	70	0·01294
30	10	60	0·00550	50	0·01142	32	16	90	0·00691	80	0·01317
30	12	70	0·00573	60	0·01166	32	50	100	0·00715	90	0·01139
30	14	90	0·00953	80	0·01045	32	52	80	0·00808	70	0·01266
30	48	100	0·00674	90	0·01076	32	54	70	0·00507	60	0·01312
30	50	80	0·00759	70	0·01226	32	56	60	0·00488	50	0·01309
30	52	70	0·00784	60	0·01269	32	58	50	0·00771	40	0·02121
30	54	60	0·00784	50	0·01265	32	60	50	0·00437	40	0·01216
30	56	50	0·00745	40	0·02054	32	62	40	0·00664	30	0·01894
30	58	50	0·00424	40	0·01179	32	64	40	0·00345	30	0·01894
30	60	40	0·00646	30	0·01850	32	66	30	0·00969	20	0·02808
30	62	30	0·00955	20	0·05548	32	68	30	0·00969	20	0·01282

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
32	70	30	0·00460	20	0·01282	34	76	30	0·00080	20	0·01242
32	72	30	0·00201	20	0·01282	34	78	20	0·00477	10	0·03489
32	74	20	0·00519	10	0·08945	34	80	20	0·00477	10	0·03489
32	76	20	0·00519	10	0·03489	36	4	30	0·00123	20	0·02180
34	0	20	0·00416	10	0·10526	36	6	30	0·00512	20	0·02180
34	2	20	0·00416	10	0·10526	36	8	30	0·00512	20	0·06369
34	4	30	0·00279	20	0·02180	36	10	40	0·00724	30	0·01517
34	6	40	0·00073	30	0·01057	36	12	50	0·00456	40	0·01695
34	8	40	0·00259	30	0·01057	36	14	50	0·00991	40	0·03458
34	10	40	0·00724	30	0·02873	36	16	70	0·00567	60	0·01116
34	12	50	0·00815	40	0·01695	36	18	80	0·00601	70	0·01789
34	14	60	0·00831	50	0·01689	36	20	100	0·00886	90	0·01498
34	16	70	0·00921	60	0·02836	36	54	90	0·00814	80	0·01925
34	52	100	0·00748	90	0·01189	36	56	80	0·00856	70	0·01353
34	54	80	0·00827	70	0·01330	36	58	70	0·00542	60	0·01392
34	56	60	0·00834	50	0·02193	36	60	60	0·00860	50	0·01359
34	58	60	0·00501	50	0·01340	36	62	50	0·00797	40	0·01235
34	60	50	0·00789	40	0·02162	36	64	40	0·00670	30	0·03493
34	62	50	0·00446	40	0·01235	36	66	40	0·00670	30	0·01894
34	64	40	0·00670	30	0·01894	36	68	40	0·00345	30	0·01894
34	66	30	0·00969	20	0·05642	36	70	30	0·00955	20	0·02808
34	68	30	0·00969	20	0·02808	36	72	30	0·00443	20	0·02808
34	70	30	0·00460	20	0·02808	36	74	30	0·00443	20	0·01242
34	72	30	0·00201	20	0·02808	36	76	30	0·00188	20	0·01242
34	74	30	0·00201	20	0·01242	36	78	20	0·00477	10	0·08491

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
36	80	20	0·00477	10	0·08491	38	78	30	0·00188	20	0·01124
36	82	20	0·00477	10	0·08491	38	80	30	0·00072	20	0·01124
36	84	20	0·00153	10	0·08491	38	82	30	0·00024	20	0·01124
36	86	20	0·00153	10	0·02864	38	84	20	0·00396	10	0·08490
38	4	20	0·00983	10	0·04334	38	86	20	0·00396	10	0·02864
38	6	20	0·00983	10	0·15170	38	88	20	0·00110	10	0·02864
38	8	30	0·00512	20	0·03242	38	90	20	0·00110	10	0·02864
38	10	40	0·00377	30	0·01517	38	92	20	0·00110	10	0·02864
38	12	40	0·00939	30	0·03581	40	8	30	0·00238	20	0·03242
38	14	50	0·00565	40	0·02030	40	10	30	0·00767	20	0·03242
38	16	60	0·00674	50	0·01164	40	12	40	0·00502	30	0·01955
38	18	70	0·00722	50	0·01253	40	14	50	0·00313	40	0·01150
38	20	90	0·00661	80	0·01130	40	16	50	0·00674	40	0·01150
38	22	110	0·00604	100	0·01012	40	18	60	0·00765	50	0·01330
38	56	100	0·00790	90	0·01237	40	20	70	0·00798	60	0·01385
38	58	80	0·00864	70	0·01379	40	22	90	0·00767	80	0·01305
38	60	70	0·00882	60	0·01401	40	58	100	0·00799	90	0·01252
38	62	60	0·00863	50	0·01366	40	60	80	0·00872	70	0·01382
38	64	50	0·00797	40	0·01235	40	62	70	0·00882	60	0·01401
38	66	50	0·00446	40	0·01235	40	64	60	0·00860	50	0·01359
38	68	40	0·00664	30	0·01894	40	66	50	0·00789	40	0·02162
38	70	30	0·00955	20	0·05548	40	68	50	0·00437	40	0·01216
38	72	30	0·00443	20	0·05548	40	70	40	0·00646	30	0·01850
38	74	30	0·00443	20	0·02678	40	72	30	0·00912	20	0·05548
38	76	30	0·00188	20	0·02678	40	74	30	0·00912	20	0·02678

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Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.		Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
40	76	30	0·00411	20	0·02678	42	74	40	0·00301	30	0·01761
40	78	30	0·00411	20	0·01124	42	76	30	0·00843	20	0·02678
40	80	30	0·00166	20	0·01124	42	78	30	0·00843	20	0·01124
40	82	30	0·00060	20	0·01124	42	80	30	0·00364	20	0·01124
40	84	20	0·00396	10	0·08490	42	82	30	0·00139	20	0·01124
40	86	20	0·00396	10	0·02864	44	8	30	0·00106	20	0·01548
40	88	20	0·00110	10	0·02864	44	10	30	0·00370	20	0·01548
40	90	20	0·00110	10	0·02864	44	12	40	0·00129	30	0·01019
4C	92	20	0·00110	10	0·02864	44	14	40	0·00330	30	0·01019
42	8	30	0·00106	20	0·03242	44	16	40	0·00330	30	0·02351
42	10	30	0·00370	20	0·03242	44	18	40	0·00749	30	0·02351
42	12	40	0·00259	30	0·01019	44	20	50	0·00883	40	0·01534
42	14	40	0·00627	30	0·01019	44	22	60	0·00938	50	0·01633
42	16	40	0·00627	30	0·02351	44	24	80	0·00590	70	0·01008
42	18	50	0·00781	40	0·01350	44	26	90	0·00606	80	0·01537
42	20	60	0·00853	50	0·01487	44	62	100	0·00790	90	0·01237
42	22	70	0·00871	60	0·01509	44	64	80	0·00856	70	0·01353
42	24	80	0·00914	70	0·02360	44	66	60	0·00834	50	0·02193
42	60	100	0·00799	90	0·01252	44	68	60	0·00488	50	0·01309
42	62	80	0·00864	70	0·01379	44	70	50	0·00745	40	0·02054
42	64	70	0·00542	60	0·01392	44	72	50	0·00404	40	0·01124
42	66	60	0·00501	50	0·01340	44	74	40	0·00575	30	0·01761
42	68	50	0·00771	40	0·02121	44	76	30	0·00843	20	0·05267
42	70	50	0·00424	40	0·01179	44	78	30	0·00843	20	0·02419
42	72	40	0·00616	30	0·01761	44	80	30	0·00364	20	0·02419

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
44	82	30	0·00139	20	0·02419	46	84	20	0·00935	10	0·17492
44	84	20	0·00935	10	0·08491	46	86	20	0·00935	10	0·07044
44	86	20	0·00935	10	0·02864	48	8	20	0·00691	10	0·07044
46	8	30	0·00045	20	0·01548	48	10	20	0·00691	10	0·07044
46	10	30	0·00171	20	0·01548	48	12	20	0·00691	10	0·07044
46	12	30	0·00506	20	0·01548	48	14	30	0·00506	20	0·02037
46	14	30	0·00506	20	0·04118	48	16	40	0·00167	30	0·01253
46	16	40	0·00330	30	0·01253	48	18	40	0·00400	30	0·01253
46	18	40	0·00749	30	0·01253	48	20	40	0·00862	30	0·02695
46	20	50	0·00508	40	0·01534	48	22	50	0·00567	40	0·01699
46	22	50	0·00978	40	0·02878	48	24	60	0·00367	50	0·01065
46	24	60	0·00620	50	0·01765	48	26	70	0·00420	60	0·01157
46	26	80	0·00665	70	0·01070	48	28	80	0·00699	70	0·01179
46	28	100	0·00627	90	0·01000	48	30	100	0·00674	90	0·01076
46	64	90	0·00814	80	0·01925	48	66	100	0·00748	90	0·01189
46	66	80	0·00827	70	0·01330	48	68	80	0·00808	70	0·01266
46	68	70	0·00507	60	0·01312	48	70	70	0·00784	60	0·01269
46	70	60	0·00784	50	0·01265	48	72	60	0·00751	50	0·01209
46	72	50	0·00711	40	0·01124	48	74	50	0·00669	40	0·01051
46	74	40	0·00575	30	0·03204	48	76	50	0·00351	40	0·01051
46	76	40	0·00575	30	0·01630	48	78	40	0·00525	30	0·01630
46	78	40	0·00274	30	0·01630	48	80	30	0·00749	20	0·04792
46	80	30	0·00749	20	0·02419	48	82	30	0·00305	20	0·04792
46	82	30	0·00305	20	0·02419	48	84	30	0·00305	20	0·02037

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value or N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value or N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
48	86	30	0·00108	20	0·02037	52	92	20	0·00691	10	0·07044
48	88	20	0·00691	10	0·07044	52	90	20	0·00691	10	0·07044
						52	88	20	0·00691	10	0·07044
50	14	30	0·00239	20	0·02037	52	86	30	0·00506	20	0·02037
50	16	30	0·00635	20	0·02037	52	84	40	0·00167	30	0·01253
50	18	30	0·00635	20	0·04792	52	82	40	0·00400	30	0·01253
50	20	40	0·00466	30	0·01459	52	80	40	0·00862	30	0·02695
50	22	40	0·00964	30	0·02980	52	78	50	0·00367	40	0·01699
50	24	50	0·00622	40	0·01842	52	76	60	0·00367	50	0·01065
50	26	60	0·00712	50	0·01142	52	74	70	0·00420	60	0·01157
50	28	70	0·00753	60	0·01217	52	72	80	0·00699	70	0·01179
50	30	80	0·00759	70	0·01226	52	70	100	0·00674	90	0·01076
50	32	100	0·00715	90	0·01139	52	34	100	0·00748	90	0·01189
50	68	100	0·00715	90	0·01139	52	32	80	0·00808	70	0·01266
50	70	80	0·00759	70	0·01226	52	30	70	0·00784	60	0·01269
50	72	70	0·00753	60	0·01217	52	28	60	0·00751	50	0·01209
50	74	60	0·00712	50	0·01142	52	26	50	0·00670	40	0·01051
50	76	50	0·00622	40	0·01842	52	24	50	0·00351	40	0·01051
50	78	40	0·00964	30	0·02880	52	22	40	0·00525	30	0·01630
50	80	40	0·00466	30	0·01459	52	20	30	0·00749	20	0·04792
50	82	30	0·00635	20	0·04792	52	18	30	0·00305	20	0·04792
50	84	30	0·00635	20	0·02037	52	16	30	0·00305	20	0·02037
50	86	30	0·00239	20	0·02037	52	14	30	0·00108	20	0·02037
50	88	20	0·00691	10	0·07044	52	12	20	0·00691	10	0·07044

*Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—*contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
54	92	30	0·00045	20	0·01548	56	88	40	0·00129	30	0·01019
54	90	30	0·00171	20	0·01548	56	86	40	0·00330	30	0·01019
54	88	30	0·00506	20	0·01548	56	84	40	0·00330	30	0·02351
54	86	30	0·00506	20	0·04118	56	82	40	0·00749	30	0·02351
54	84	40	0·00330	30	0·01253	56	80	50	0·00883	40	0·01534
54	82	40	0·00749	30	0·01253	56	78	60	0·00938	50	0·01633
54	80	50	0·00508	40	0·01534	56	76	80	0·00590	70	0·01008
54	78	50	0·00978	40	0·02878	56	74	90	0·00606	80	0·01537
54	76	60	0·00620	50	0·01765	56	38	100	0·00790	90	0·01237
54	74	80	0·00665	70	0·01070	56	36	80	0·00856	70	0·01359
54	72	100	0·00627	90	0·01000	56	34	60	0·00834	50	0·02193
54	36	90	0·00814	80	0·01925	56	32	60	0·00488	50	0·01309
54	34	80	0·00827	70	0·01330	56	30	50	0·00745	40	0·02054
54	32	70	0·00507	60	0·01312	56	28	50	0·00404	40	0·01124
54	30	60	0·00784	50	0·01265	56	26	40	0·00575	30	0·01761
54	28	50	0·00711	40	0·01124	56	24	30	0·00843	20	0·05267
54	26	40	0·00575	30	0·03204	56	22	30	0·00843	20	0·02419
54	24	40	0·00575	30	0·01630	56	20	30	0·00364	20	0·02419
54	22	40	0·00274	30	0·01630	56	18	30	0·00139	20	0·02419
54	20	30	0·00749	20	0·02419	56	16	20	0·00935	10	0·08490
54	18	30	0·00305	20	0·02419	56	14	20	0·00935	10	0·02864
54	16	20	0·00935	10	0·17492	58	92	30	0·00106	20	0·03242
54	14	20	0·00935	10	0·07044	58	90	30	0·00370	20	0·03242
56	92	30	0·00106	20	0·01548	58	88	40	0·00259	30	0·01019
56	90	30	0·00370	20	0·01548	58	86	40	0·00627	30	0·01019

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.
58	84	40	0·00627	30	0·02351
58	82	50	0·00781	40	0·01350
58	80	60	0·00853	50	0·01487
58	78	70	0·00871	60	0·01509
58	76	80	0·00914	70	0·02360
58	40	100	0·00799	90	0·01252
58	38	80	0·00874	70	0·01379
58	36	70	0·00542	60	0·01392
58	34	60	0·00501	50	0·01340
58	32	50	0·00771	40	0·02121
58	30	50	0·00424	40	0·01179
58	28	40	0·00616	30	0·01761
58	26	40	0·00301	30	0·01761
58	24	30	0·00843	20	0·02678
58	22	30	0·00843	20	0·01124
58	20	30	0·00364	20	0·01124
58	18	30	0·00139	20	0·01124
58	16	20	0·00396	10	0·08490
60	92	30	0·00238	20	0·03242
60	90	30	0·00767	20	0·03242
60	88	40	0·00502	30	0·01955
60	86	50	0·00313	40	0·01150
60	84	50	0·00674	40	0·01150
60	82	60	0·00765	50	0·01330
60	80	70	0·00798	60	0·01385

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.
60	78	90	0·00767	80	0·01305
60	42	100	0·00799	90	0·01242
60	40	80	0·00872	70	0·01382
60	38	70	0·00882	60	0·01401
60	36	60	0·00860	50	0·01359
60	34	50	0·00789	40	0·02162
60	32	50	0·00437	40	0·01216
60	30	40	0·00646	30	0·01850
60	28	30	0·00912	20	0·05548
60	26	30	0·00912	20	0·02678
60	24	30	0·00411	20	0·02678
60	22	30	0·00411	20	0·01124
60	20	30	0·00166	20	0·01124
60	18	30	0·00060	20	0·01124
60	16	20	0·00396	10	0·08490
60	14	20	0·00396	10	0·02864
62	96	20	0·00983	10	0·04334
62	94	20	0·00983	10	0·15170
62	92	30	0·00512	20	0·03242
62	90	40	0·00377	30	0·01517
62	88	40	0·00939	30	0·03581
62	86	50	0·00565	40	0·02030
62	84	60	0·00674	50	0·01164
62	82	70	0·00722	60	0·01253
62	80	90	0·00661	80	0·01130

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.
62	78	110	0·00604	100	0·01012
62	44	100	0·00790	90	0·01237
62	42	80	0·00864	70	0·01379
62	40	70	0·00882	60	0·01401
62	38	60	0·00863	50	0·01366
62	36	50	0·00797	40	0·01235
62	34	50	0·00446	40	0·01235
62	32	40	0·00664	30	0·01894
62	30	30	0·00955	20	0·05548
62	28	30	0·00443	20	0·05548
62	26	30	0·00443	20	0·02678
62	24	30	0·00188	20	0·02678
62	22	30	0·00188	20	0·01124
62	20	30	0·00072	20	0·01124
62	18	30	0·00024	20	0·01124
62	16	20	0·00396	10	0·08490
62	14	20	0·00396	10	0·02864
64	96	30	0·00123	20	0·02180
64	94	30	0·00512	20	0·02180
64	92	30	0·00512	20	0·06369
64	90	40	0·00724	30	0·01517
64	88	50	0·00456	40	0·01695
64	86	50	0·00991	40	0·03458
64	84	70	0·00567	60	0·01116
64	82	80	0·00601	70	0·01789

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.
64	80	100	0·00886	90	0·01498
64	46	90	0·00814	80	0·01925
64	44	80	0·00856	70	0·01353
64	42	70	0·00542	60	0·01392
64	40	60	0·00860	50	0·01359
64	38	50	0·00797	40	0·01235
64	36	40	0·00670	30	0·03493
64	34	40	0·00670	30	0·01894
64	32	40	0·00345	30	0·01894
64	30	30	0·00955	20	0·02808
64	28	30	0·00443	20	0·02808
64	26	30	0·00443	20	0·01242
64	24	30	0·00188	20	0·01242
64	22	20	0·00477	10	0·08491
64	20	20	0·00477	10	0·08491
66	100	20	0·00416	10	0·10526
66	98	20	0·00416	10	0·10526
66	96	30	0·00279	20	0·02180
66	94	40	0·00073	30	0·01057
66	92	40	0·00259	30	0·01057
66	90	40	0·00724	30	0·02873
66	88	50	0·00815	40	0·01695
66	86	60	0·00831	50	0·01689
66	84	70	0·00921	60	0·02836
66	48	100	0·00748	90	0·01189

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		HIGHER VALUE or N.		LOWER VALUE or N.		Comparison between, per cent.		HIGHER VALUE or N.		LOWER VALUE or N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
66	46	80	0·00827	70	0·01330	68	46	70	0·00507	60	0·01312
66	44	60	0·00834	50	0·02193	68	44	60	0·00488	50	0·01309
66	42	60	0·00501	50	0·01340	68	42	50	0·00771	40	0·02121
66	40	50	0·00789	40	0·02162	68	40	50	0·00437	40	0·01216
66	38	50	0·00446	40	0·01235	68	38	40	0·00664	30	0·01894
66	36	40	0·00670	30	0·01894	68	36	40	0·00345	30	0·01894
66	34	30	0·00969	20	0·05642	68	34	30	0·00969	20	0·02808
66	32	30	0·00969	20	0·02808	68	32	30	0·00969	20	0·01282
66	30	30	0·00160	20	0·02808	68	30	30	0·00460	20	0·01282
66	28	30	0·00201	20	0·02808	68	28	30	0·00201	20	0·01282
66	26	30	0·00201	20	0·01242	68	26	20	0·00519	10	0·08915
66	24	30	0·00080	20	0·01242	68	24	20	0·00519	10	0·03489
66	22	20	0·00477	10	0·03489						
66	20	20	0·00177	10	0·03489	70	100	30	0·00097	20	0·01010
						70	98	30	0·00609	20	0·01010
68	100	30	0·00010	20	0·01010	70	96	30	0·00609	20	0·01571
68	98	30	0·00279	20	0·01010	70	94	40	0·00322	30	0·02095
68	96	30	0·00279	20	0·01571	70	92	40	0·00988	30	0·02095
68	94	10	0·00156	30	0·01057	70	90	60	0·00550	50	0·01112
68	92	10	0·00514	30	0·01057	70	88	70	0·00573	60	0·01166
68	90	50	0·00641	40	0·01343	70	86	90	0·00953	80	0·01015
68	88	60	0·00689	50	0·01417	70	52	100	0·00674	90	0·01076
68	86	80	0·00106	70	0·01294	70	50	80	0·00759	70	0·01226
68	84	90	0·00691	80	0·01317	70	48	70	0·00784	60	0·01269
68	50	100	0·00715	90	0·01139	70	46	60	0·00784	50	0·01265
68	48	80	0·00808	70	0·01266	70	44	50	0·00745	40	0·02051

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value or N.		Lower value or N.		Comparison between, per cent.		Higher value or N.		Lower value or N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
70	42	50	0·00424	40	0·01179	72	38	30	0·00443	20	0·05548
70	40	40	0·00646	30	0·01850	72	36	30	0·00443	20	0·02808
70	38	30	0·00955	20	0·05548	72	34	30	0·00201	20	0·02808
70	36	30	0·00955	20	0·02808	72	32	30	0·00201	20	0·01282
70	34	30	0·00460	20	0·02808	72	30	30	0·00085	20	0·01282
70	32	30	0·00460	20	0·01282	72	28	30	0·00033	20	0·01282
70	30	30	0·00206	20	0·01282	72	26	20	0·00519	10	0·08945
70	28	30	0·00085	20	0·01282	74	100	30	0·00229	20	0·02356
70	26	20	0·00519	10	0·08945	74	98	40	0·00346	30	0·01285
72	100	30	0·00229	20	0·01010	74	96	50	0·00191	40	0·01260
72	98	40	0·00163	30	0·01285	74	94	50	0·00611	40	0·01260
72	96	40	0·00647	30	0·01285	74	92	60	0·00742	50	0·01551
72	94	40	0·00647	30	0·03986	74	90	80	0·00642	70	0·01297
72	92	50	0·00870	40	0·01837	74	88	100	0·00916	90	0·01758
72	90	60	0·00951	50	0·01976	74	56	90	0·00606	80	0·01537
72	88	70	0·00952	60	0·01926	74	54	80	0·00665	70	0·01070
72	86	110	0·00617	100	0·01164	74	52	70	0·00420	60	0·01157
72	54	110	0·00627	90	0·01000	74	50	60	0·00712	50	0·01142
72	52	80	0·00699	70	0·01179	74	48	50	0·00670	40	0·01051
72	50	70	0·00753	60	0·01217	74	46	40	0·00575	30	0·03204
72	48	60	0·00751	50	0·01209	74	44	40	0·00575	30	0·01761
72	46	50	0·00711	40	0·01124	74	42	40	0·00301	30	0·01761
72	44	50	0·00404	40	0·01124	74	40	30	0·00912	20	0·02678
72	42	40	0·00616	30	0·01761	74	38	30	0·00443	20	0·02678
72	40	20	0·00912	20	0·05548	74	36	30	0·00443	20	0·01242

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.		Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OR N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
74	34	30	0·00201	20	0·01242	78	96	50	0·00734	40	0·02381
74	32	20	0·00519	10	0·08945	78	94	70	0·00598	60	0·01694
						78	92	80	0·00680	70	0·02820
76	100	30	0·00527	20	0·02356	78	62	110	0·00604	100	0·01012
76	98	40	0·00346	30	0·02616	78	60	90	0·00767	80	0·01305
76	96	50	0·00379	40	0·01260	78	58	70	0·00871	60	0·01509
76	94	60	0·00973	50	0·01130	78	56	60	0·00938	50	0·01633
76	92	80	0·00400	70	0·01053	78	54	50	0·00978	40	0·02878
76	90	90	0·00842	80	0·01671	78	52	50	0·00567	40	0·01699
76	58	80	0·00914	70	0·02360	78	50	40	0·00964	30	0·02980
76	56	80	0·00590	70	0·01008	78	48	40	0·00525	30	0·01630
76	54	60	0·00620	50	0·01765	78	46	40	0·00274	30	0·01630
76	52	60	0·00367	50	0·01065	78	44	30	0·00843	20	0·02419
76	50	50	0·00622	40	0·01842	78	42	30	0·00843	20	0·01124
76	48	50	0·00351	40	0·01051	78	40	30	0·00411	20	0·01124
76	46	40	0·00575	30	0·01630	78	38	30	0·00188	20	0·01124
76	44	30	0·00843	20	0·05267	78	36	20	0·00477	10	0·08491
76	42	30	0·00843	20	0·02678	80	100	40	0·00265	30	0·01186
76	40	30	0·00411	20	0·02678	80	98	50	0·00389	40	0·01444
76	38	30	0·00188	20	0·02678	80	96	60	0·00419	50	0·01387
76	36	30	0·00188	20	0·01242	80	94	80	0·00877	70	0·01049
76	34	30	0·00080	20	0·01242	80	92	110	0·00947	100	0·01183
76	32	20	0·00519	10	0·03489	80	64	100	0·00886	90	0·01498
78	100	30	0·00527	20	0·05301	80	62	90	0·00661	80	0·01130
78	98	40	0·00716	30	0·02616	80	60	70	0·00798	60	0·01385

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
80	58	60	0·00853	50	0·01487	82	48	30	0·00305	20	0·04792
80	56	50	0·00883	40	0·01534	82	46	30	0·00305	20	0·02419
80	54	50	0·00508	40	0·01534	82	44	30	0·00139	20	0·02419
80	52	40	0·00862	30	0·02695	82	42	30	0·00139	20	0·01124
80	50	40	0·00466	30	0·01459	82	40	30	0·00060	20	0·01124
80	48	30	0·00749	20	0·04792	82	38	30	0·00024	20	0·01124
80	46	30	0·00749	20	0·02419	82	36	20	0·00477	10	0·08491
80	44	30	0·00364	20	0·02419						
80	42	30	0·00364	20	0·01124	84	100	50	0·00289	40	0·01277
80	40	30	0·00166	20	0·01124	84	98	60	0·00419	50	0·01543
80	38	30	0·00072	20	0·01124	84	96	100	0·00404	90	0·01153
80	36	20	0·00477	10	0·08491	84	68	90	0·00691	80	0·01317
						84	66	70	0·00921	60	0·02836
82	100	40	0·00587	30	0·02609	84	64	70	0·00567	60	0·01116
82	98	50	0·00783	40	0·02838	84	62	60	0·00674	50	0·01164
82	96	60	0·00794	50	0·02556	84	60	50	0·00674	40	0·01150
82	94	90	0·00924	80	0·02415	84	58	40	0·00627	30	0·02351
82	66	90	0·00849	80	0·01453	84	56	40	0·00330	30	0·02351
82	64	80	0·00601	70	0·01789	84	54	40	0·00330	30	0·01253
82	62	70	0·00722	60	0·01253	84	52	40	0·00167	30	0·01253
82	60	60	0·00765	50	0·01330	84	50	30	0·00635	20	0·02037
82	58	50	0·00781	40	0·01350	84	48	30	0·00305	20	0·02037
82	56	40	0·00749	30	0·02351	84	46	20	0·00935	10	0·17492
82	54	40	0·00749	30	0·01253						
82	52	40	0·00400	30	0·01253	86	100	50	0·00624	40	0·01277
82	50	30	0·00635	20	0·04792	86	98	70	0·00442	60	0·01609

*Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—*contd.

Comparison between, per cent.		Higher value of N.		Lower value of N.		Comparison between, per cent.		Higher value of N.		Lower value of N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
86	72	110	0·00617	100	0·01164	88	58	40	0·00259	30	0·01019
86	70	90	0·00953	80	0·01045	88	56	40	0·00129	30	0·01019
86	68	80	0·00406	70	0·01294	88	54	30	0·00506	20	0·01548
86	66	60	0·00831	50	0·01689	88	52	20	0·00691	10	0·07044
86	64	50	0·00991	40	0·03458						
86	62	50	0·00565	40	0·02030	90	100	70	0·00668	60	0·01371
86	60	50	0·00313	40	0·01150	90	98	110	0·00938	100	0·01652
86	58	40	0·00627	30	0·01019	90	76	90	0·00842	80	0·01671
86	56	40	0·00330	30	0·01019	90	74	80	0·00642	70	0·01297
86	54	30	0·00506	20	0·04118	90	72	60	0·00951	50	0·01976
86	52	30	0·00506	20	0·02037	90	70	60	0·00550	50	0·01142
86	50	30	0·00239	20	0·02037	90	68	50	0·00641	40	0·01343
86	48	30	0·00108	20	0·02037	90	66	40	0·00724	30	0·02873
86	46	20	0·00935	10	0·07044	90	64	40	0·00724	30	0·01517
						90	62	40	0·00377	30	0·01517
88	100	60	0·00649	50	0·01333	90	60	30	0·00767	20	0·03242
88	98	90	0·00899	80	0·01585	90	58	30	0·00370	20	0·03242
88	74	100	0·00916	90	0·01758	90	56	30	0·00370	20	0·01548
88	72	70	0·00952	60	0·01926	90	54	30	0·00171	20	0·01548
88	70	70	0·00573	60	0·01166	90	52	20	0·00691	10	0·07044
88	68	60	0·00689	50	0·01417						
88	66	50	0·00815	40	0·01695	92	100	90	0·00692	80	0·01418
88	64	50	0·00456	40	0·01695	92	80	110	0·00947	100	0·01183
88	62	40	0·00939	30	0·03581	92	78	80	0·00680	70	0·02820
88	60	40	0·00502	30	0·01955	92	76	80	0·00400	70	0·01053

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—contd.

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.		Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
92	74	60	0·00742	50	0·01551	96	84	100	0·00404	90	0·01153
92	72	50	0·00870	40	0·01837	96	82	60	0·00794	50	0·02556
92	70	40	0·00988	30	0·02095	96	80	60	0·00419	50	0·01387
92	68	40	0·00514	30	0·01057	96	78	50	0·00734	40	0·02381
92	66	40	0·00259	30	0·01057	96	76	50	0·00379	40	0·01260
92	64	30	0·00512	20	0·06369	96	74	50	0·00191	40	0·01260
92	62	30	0·00512	20	0·03242	96	72	40	0·00647	30	0·01285
92	60	30	0·00238	20	0·03242	96	70	30	0·00609	20	0·04574
92	58	30	0·00106	20	0·03242	96	68	30	0·00279	20	0·04574
92	56	30	0·00106	20	0·01548	96	66	30	0·00279	20	0·02180
92	54	30	0·00045	20	0·01548	96	64	30	0·00123	20	0·02180
92	52	20	0·00691	10	0·07044	96	62	20	0·00983	10	0·04334
94	100	110	0·00708	100	0·01447						
94	82	90	0·00924	80	0·02415	98	90	110	0·00938	100	0·01652
94	80	80	0·00877	70	0·01049	98	88	90	0·00899	80	0·01585
94	78	70	0·00598	60	0·01694	98	86	70	0·00442	60	0·01609
94	76	60	0·00973	50	0·01130	98	84	60	0·00419	50	0·01543
94	74	50	0·00611	40	0·01260	98	82	50	0·00783	40	0·02838
94	72	40	0·00647	30	0·03986	98	80	50	0·00389	40	0·01444
94	70	40	0·00322	30	0·02095	98	78	40	0·00716	30	0·02616
94	68	40	0·00156	30	0·01057	98	76	40	0·00346	30	0·02616
94	66	40	0·00073	.30	0·01057	98	74	40	0·00346	30	0·01285
94	64	30	0·00512	20	0·02180	98	72	40	0·00163	30	0·01285
94	62	20	0·00983	10	0·15170	98	70	30	0·00609	20	0·01010

Size of samples necessary to bring out significant difference between pairs of percentage responses to two methods of treatment—concld.

Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.		Comparison between, per cent.		HIGHER VALUE OF N.		LOWER VALUE OF N.	
		N.	Corresponding probability.	N.	Corresponding probability.			N.	Corresponding probability.	N.	Corresponding probability.
98	68	30	0·00279	20	0·01010	100	82	40	0·00587	30	0·02609
98	66	20	0·00416	10	0·10526	100	80	40	0·00265	30	0·01186
						100	78	30	0·00527	20	0·05301
100	94	110	0·00708	100	0·01447	100	76	30	0·00527	20	0·02356
100	92	90	0·00692	80	0·01418	100	74	30	0·00229	20	0·02356
100	90	70	0·00668	60	0·01371	100	72	30	0·00229	20	0·01010
100	88	60	0·00649	50	0·01333	100	70	30	0·00097	20	0·01010
100	86	50	0·00624	40	0·01277	100	68	30	0·00040	20	0·01010
100	84	50	0·00289	40	0·01277	100	66	20	0·00416	10	0·10526

The following has been received for publication :—

ANNOUNCEMENT

Of the Francis Amory Septennial Prize of the American Academy of Arts and Sciences under the Will of Francis Amory.

In compliance with the provisions of the Will of the late Francis Amory, the American Academy of Arts and Sciences, as Trustee of a fund given by the testator, announces a prize to be known as 'The Francis Amory Septennial Prize' to be awarded for conspicuously meritorious work performed during the immediately preceding septennial period, 'through experiment, study or otherwise, in the treatment and cure of diseases and derangement of human sexual generative organs in general, and more especially for the cure, prevention or relief of the retention of urine, cystitis. prostatitis, etc.' While the donor wished especially to reward the discovery of any new method of treatment, he expressly authorized that the prize might be given to any author who might have contributed any theoretical or practical treatise of extraordinary exceptional value and merit on the anatomy of said organs or the treatment of their diseases.

If there shall appear work of a quality to warrant it, the first award will be made in 1940. The total amount will exceed $10,000 which may be divided at the discretion of the Academy among several nominees. While formal nominations are not expected and no essays or treatises in direct competition for the prize are desired, the Committee invites suggestions looking toward the wise performance of their duty. Communications on this subject should reach the Committee not later than 15th May, 1940, and should be addressed in care of the American Academy of Arts and Sciences, 28, Newbury Street, Boston, Mass., U. S. A. The members of the Committee on the Francis Amory Septennial Prize are: Dr. Roger I. Lee, Chairman; Dr. Walter B. Cannon, Dr. David Cheever, Prof. Leigh Hoadley, Dr. William C. Quinby, Dr. E. E. Tyzzer and Dr. Soma Weiss, Secretary.

—Editor,
Indian Journal of Medical Research.

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